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14. ABSTRACT
Falls and debilitating bone fractures are a major problem for veterans, and more than 40,000 veterans suffered hip fractures from 2000-2002. Falls are the main etiological factor in more than 90% of fractures, and so treatments that can improve muscle strength while at the same time increasing bone mass will significantly reduce fracture-related morbidity and mortality. Myostatin is a factor that induces muscle wasting and suppresses bone formation. Our data collected thus far demonstrate i) myostatin suppresses proliferation of aged, but not young, myoblasts, ii) myostatin is elevated with age in muscles composed primarily of slow-twitch fibers (e.g. soleus), and iii) myostatin increases muscle mass and muscle fiber size in aged mice. These findings suggest that myostatin inhibitors may have potential for suppressing muscle wasting and improving muscle repair in older individuals, but their effect on bone may be less significant.

15. SUBJECT TERMS
Aging; Osteoporosis; Fractures

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Table of Contents

Introduction

Loss of muscle mass with age is implicated in age-related bone loss, and muscle frailty contributes to an increased incidence of falls and fractures. Yet, the molecular mechanisms underlying age-related muscle wasting, and the ability of muscle to promote bone formation and fracture healing, are unknown. We have focused our research on the role of myostatin (GDF-8) in muscle-bone interactions in order to develop more effective treatment and prevention strategies for muscle injury, frailty, and bone fracture. We have previously shown that myostatin deficiency increases bone strength and biomineralization throughout the skeleton, and that a new myostatin inhibitor (propeptide) increases both muscle mass and bone formation (Hamrick et al., 2007, 2010; Elkasrawy and Hamrick, 2010). Our research therefore suggests that myostatin is a key factor regulating both myogenesis and osteogenesis. Although some studies have found no association between age and myostatin transcript levels in skeletal muscle (Marcell et al., 2001), others reveal a marked elevation in skeletal muscle myostatin expression with aging in humans (Leger et al., 2008). Additional research indicates that circulating levels of myostatin increase with age in men and women, and are highest in people aged 60-90 (Yarasheski et al., 2002). The latter finding suggests that myostatin is implicated in the sarcopenia of aging, hence myostatin inhibitors are likely to be useful pharmacological agents for treating age-related muscle atrophy as well as bone loss.

The goal of our CDMRP-sponsored research is to better characterize myostatin's role in age-related bone loss, so that targeted therapies to prevent bone fractures by enhancing muscle and bone strength can be developed. We hypothesize that the expression of myostatin and its receptor are elevated with aging in bone and muscle, which antagonizes the osteogenic and myogenic capacity of stem cells in these tissues, but that myostatin inhibitors will reverse this age-related decline in musculoskeletal function. Year 1 of the project was to determine how the expression of myostatin and its antagonist follistatin change with age in musculoskeletal tissues, whereas the goal of year 2 was to determine the effects of myostatin on anabolic pathways in primary bone-derived stromal cells and skeletal muscle myoblasts in vitro. Year 3 studies tested the hypothesis that a myostatin inhibitor could enhance muscle and bone mass in aged animals in vivo. Year 4 (extension) examined the effects of aging on myostatin signaling in human bone marrow stem cells, and the effects of a myostatin inhibitor on behavioral assessments of musculoskeletal function in aged mice.

Findings to date demonstrate that myostatin is elevated in skeletal muscle of aged mice, but only in slow- but not fast-twitch muscles. Myostatin is also elevated in bone marrow from aged mice, whereas in patient samples activin A increases with age more so than myostatin. Myostatin suppressed proliferation of aged, but not young, myoblasts, and also increased the expression of differentiation markers in myoblasts from aged mice. Myostatin did not dramatically alter osteogenic differentiation of either young or aged bone marrow stromal cells; however, myostatin does have a significant inhibitory effect on proliferation of these stem cells. Our new in vivo data using a myostatin inhibitor (propeptide) show that blocking myostatin function in vivo increases muscle mass, grip strength, and fiber size in aged mice, but does not alter bone mass, strength, or parameters of bone formation & resorption. These data suggest that targeting myostatin may have significant therapeutic potential for improving muscle function in older adults, perhaps leading to the prevention of falls and fractures.

Body
Aim 1 (months 1-12). Determine how the expression of myostatin, its receptor, and the myostatin antagonist follistatin change with age in musculoskeletal tissues.

Task 1. Human bone marrow aspirates will be collected from relatively young (18-30) and older (50-70) patients in the MCG orthopaedic clinic. Samples from younger patients are collected as waste by-products during ACL reconstructions, whereas those from older patients are discarded during total knee and hip replacement surgery.

We investigated age-related changes in the activin A-myostatin-follistatin system using bone marrow samples from young (<50 years, n=7) and older (>70 years, n=10) knee arthroplasty patients. Supernatant samples were analyzed using ELISA. Results indicate that follistatin and myostatin levels are not significantly altered with age in human bone marrow supernatants, whereas activin A levels increased increased by more than 120% in human bone marrow (Fig. 1). The marked increase in activin A levels with age in the patient samples was associated with a similar increase in the activin A: follistatin ratio.
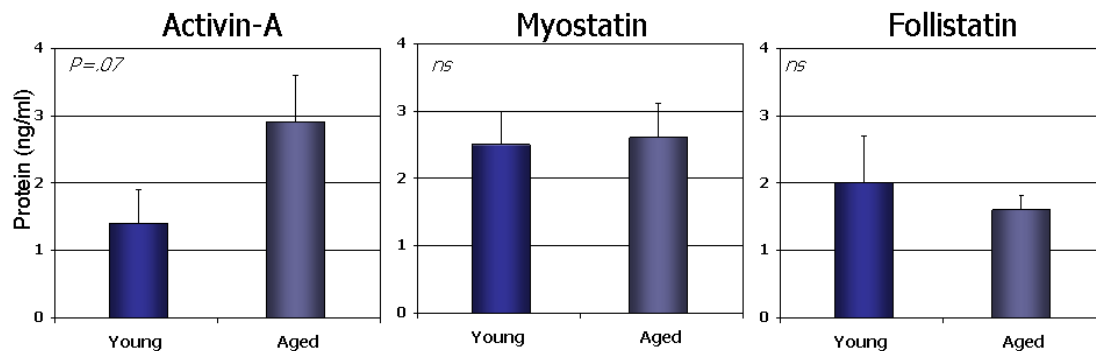

Figure 1. *Protein levels (right) of activin A, myostatin, and follistatin in bone marrow supernatants from young (<50 years) and older (>70 years) knee arthroplasty patients determined using ELISA assays.*

We also examined gene expression in bone marrow-derived stem cells to explore age-associated changes in expression of the myostatin receptor (ActRIIB), to determine how this may impact sensitivity of bone cells to the factors shown in Figure 1. Results indicate that, consistent with the data shown below in Figure 4, expression of the myostatin (and activin) receptor increases with age in BMSCs (Fig. 2).
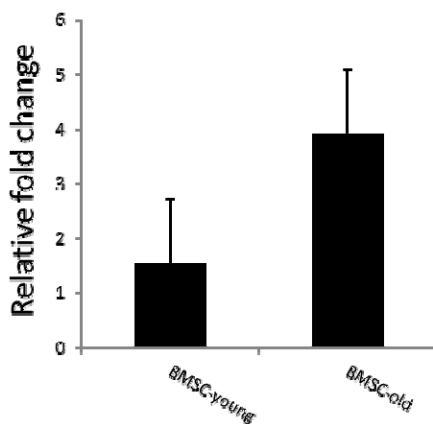

Figure 2. *Relative gene expression in BMSCs harvested from young (<50 years) and older (>70 years) knee arthroplasty patients (n=3 per group) determined using real-time PCR.*

Task 1 status: Complete.

Task 2. Bone aspirates and muscle samples are collected from mice 12, 18 and 24 months of age, 15 mice per age group (total = 45 mice).

Protein levels of activin A, myostatin, and follistatin in skeletal muscle and bone marrow of young and aged mice were determined using ELISA. We excluded the 18 mo age group because we found in PCR assays that this group was consistently an outlier. ANOVAs performed on activin A and follistatin normalized for total protein, and the ratio of normalized activin A: follistatin, revealed no significant changes with age in either the soleus or extensor digitorum longus muscles. Levels of normalized myostatin showed a slight but non-significant decrease in the EDL with age (Fig. 3A), whereas SOL showed a significant increase in normalized myostatin with age (Fig. 3A). Likewise, the ratio of normalized myostatin: follistatin

showed a significant decrease with age in EDL (Fig. 3B), but a significant increase with age in SOL (Fig. 3B). Two-factor ANOVA with age (12 or 24 mo) and muscle (EDL or SOL) as the two factors showed significant age*muscle interaction effects for both normalized myostatin (p<.05) and the myostatin: follistatin ratio (p<.01), with myostatin levels decreasing with age in the EDL but increasing with age in SOL.
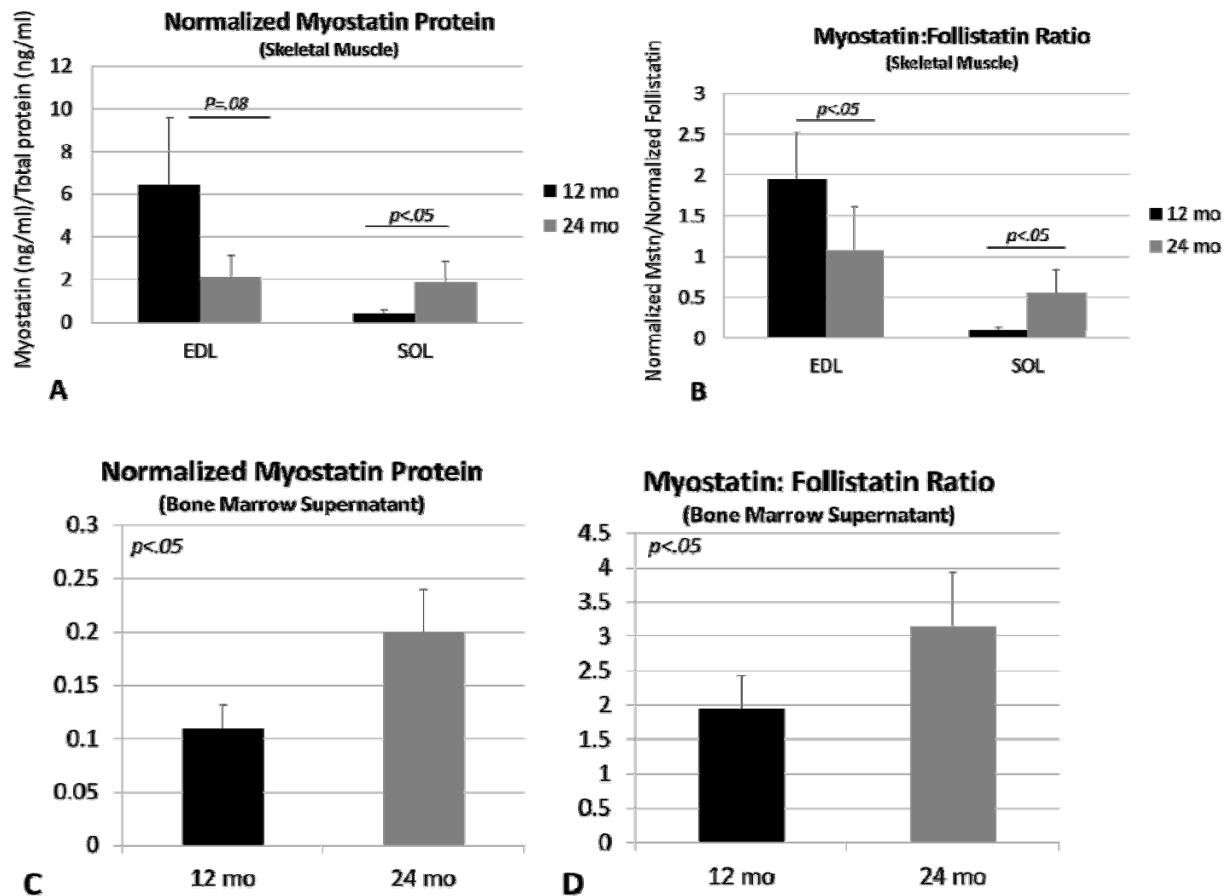

Figure 3. *Protein levels measured using ELISA of myostatin (A; normalized by BCA) and relative to follistatin (B) in the extensor digitorum longus muscle (EDL) and soleus muscle (SOL) of young (12 mo) and aged (24 mo) mice. Protein levels measured using ELISA of myostatin (C; normalized by BCA) and relative to follistatin (D) in bone marrow supernatant of young (12 mo) and aged (24 mo) mice.*

Comparisons of normalized protein levels obtained from bone marrow supernatant revealed no significant differences between older and young mice for activin A, follistatin, or the activin A: follistatin ratio. Normalized myostatin is significantly increased in mouse bone marrow with increasing age (Fig. 3C), as is the ratio of normalized myostatin: follistatin (Fig. 3D). Gene expression data showed slight increases in expression of the myostatin receptor (ActRIIB) with age in both soleus muscles and BMSCs of mice (Fig. 4).
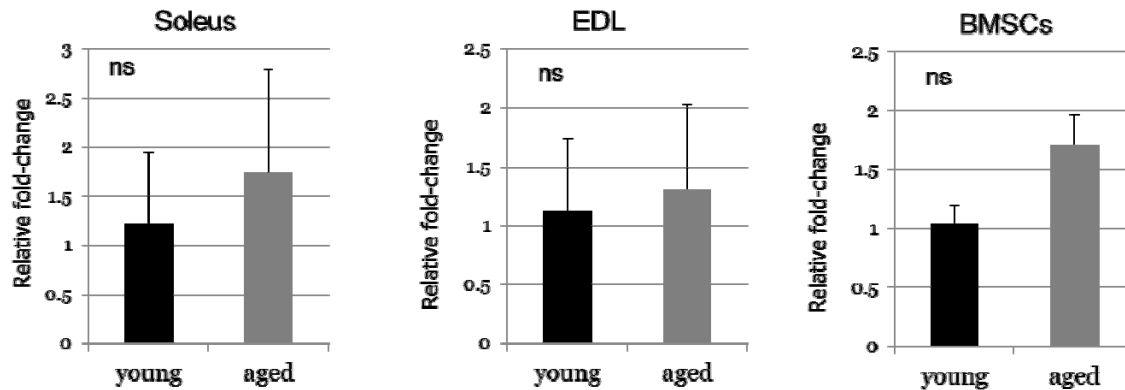
Figure 4. *Expression of the myostatin receptor (ActRIIB, or Acvr2b) in the soleus muscle (left graph), extensor digitorum longus muscle (EDL, middle graph), and in bone marrow stromal cells (BMSCs, right graph) from young (6-12 mo) and aged (24 mo) mice. Expression shows a slight but non-significant increase with age in muscle and bone tissues.*

Task 2 status. Complete.

Milestone 1: Age-related changes in the expression of myostatin, its receptor, and the myostatin antagonist follistatin in muscle and bone tissues will be defined. Milestone 1 has been reached, with the conclusion that myostatin levels in bone marrow and slow twitch muscle fibers increase slightly with age, as does expression of the myostatin receptor in BMSCs of both humans and mice. Activin A levels also increase with age in human bone marrow, but as shown below this does not have a detrimental effect on bone marrow stem cells and may actually enhance their proliferation.

Aim 2 (months 12-24). Determine the effects of myostatin on anabolic pathways in primary bone-derived stromal cells and skeletal muscle myoblasts in vitro.

Task 1 (months 12-24). Human bone marrow stromal cells from young and aged patients have been collected during year 1 for specific aim 1. In years 2 and 3 these cells will be treated with myostatin, and myostatin inhibitors, in dose-response studies.

Results demonstrate that myostatin treatment of BMSCs actually increases proliferation, which is similar to the effect observed with activin treatment of mouse myoblasts discussed below. The lower dose of propeptide also increased proliferation, although this effect was not seen at the higher dose. Age of donors did not affect the proliferative response (Fig.5).
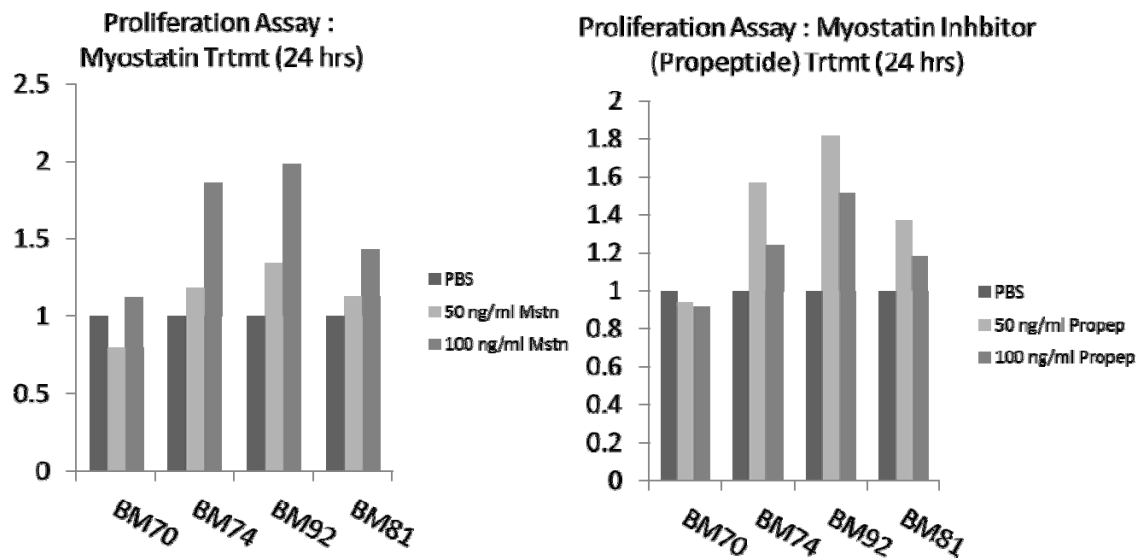
Figure 5. *Proliferation assays of primary human BMSCs isolated from young and aged patients treated with myostatin (left graph) or myostatin inhibitor (right graph). BM70=18 yrs, BM74=40 yrs, BM92=72yrs, BM81=74 yrs.*

We also examined the effects of myostatin and myostatin inhibitor on BMSCs in vitro, and found that these treatments had no impact on expression of osteogenic factors whether young or old cells are used (Fig. 6).
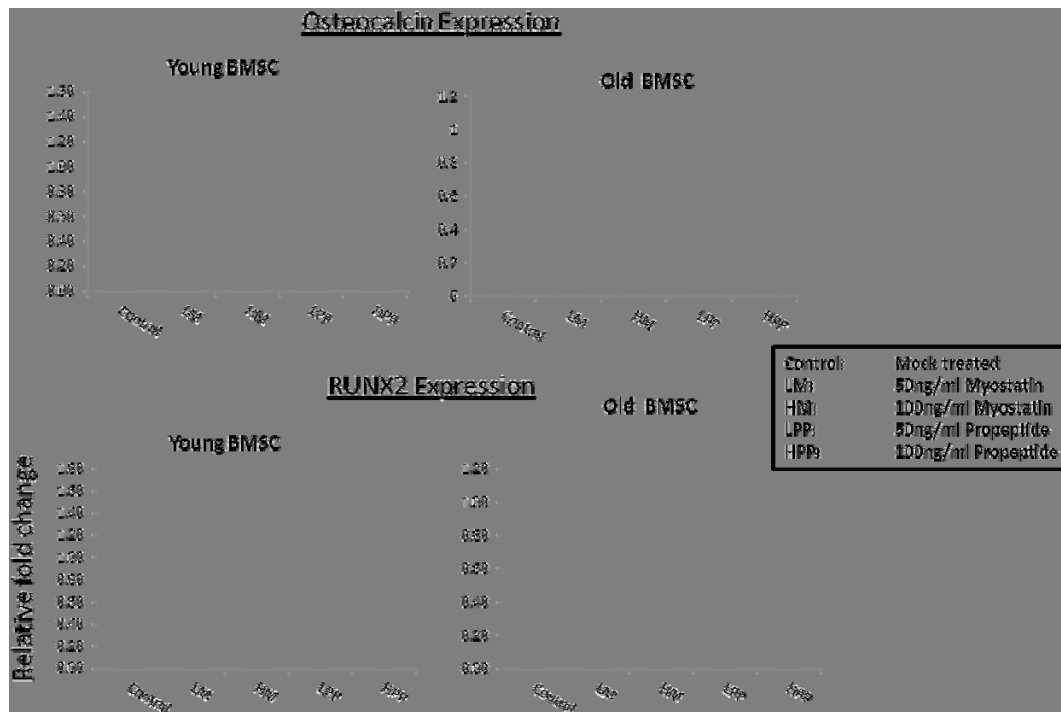

Figure 6. *Expression of the osteogenic markers osteocalcin (top) and Cbfa1/runx2 (bottom) after treatment with either myostatin or a myostatin inhibitor in young and old BMSCs. Altered myostatin signaling does not have a significant impact on these factors.*

<u>Task 1 status</u>: Complete.

6

<u>Task 2</u>. Primary myoblasts and BMSCs from young and aged mice will have been collected during year 1 for specific aim 1. In year two these cells will be treated with myostatin, and myostatin inhibitors, in dose-response studies.

Primary myoblast experiments for aim 2, task 2: For culture and treatment of primary myoblasts, tibialis anterior muscles were dissected and placed in sterile PBS. The muscle was minced with a sterile scalpel under aseptic conditions. Minced muscle was digested in 0.2% collagenase type II (Gibco) for 1 hour with frequent shaking followed by digestion in 1x trypsin for 30 minutes. The slurry was pelleted and trypsin supernatant removed. The slurry was re-suspended in proliferation medium. Upon completion of enzymatic digest, slurry was poured over a 70µm cell strainer (Fisher) to remove any remaining connective tissue. The cells were then added to collagen type I (BD Bioscience) coated T-25 flasks. Primary myoblasts were allowed to attach for 72 hours. Cells were then maintained in proliferation medium (PM): DMEM (Hyclone) supplemented with 10% fetal bovine serum, 10% horse serum, 1% penicillin / streptomycin, and 0.5% chick embryo extract (Sera Labs U.K.). Medium was changed every 48 hours until T-25 flask was confluent. Once confluent, cells were trypsinized and counted using NucleoCounter (New Brunswick Scientific). Cells were then plated in a 96 well plate at 5,000 cells/ cm2. Cells were allowed to attach in proliferation medium for 48 hours. Proliferation medium was removed, cells washed with PBS , and DMEM supplemented with 1% I.T.S was added followed by either control (PBS) or high or low dose activin A (50 ng/ml, 100 ng/ml), follistatin (100 ng/ml, 1000 ng/ml) or myostatin (100 ng/ml, 1000 ng/ml) (R&D Systems, Minneapolis). Doses follow those utilized by He et al. (2005) for activin A and Zhu et al. (2007) for myostatin and follistatin. After 24 hours of treatment, MTT reagent was added according to manufacturer's protocol and O.D. was determined 2 hours later.

For differentiation assays, cells were isolated and cultured for one week until confluent as described above. Cells were then trypsinized and plated in 12 well plates at 5,000 cells/ml and allowed to attach overnight in proliferation medium. PM was removed, cells washed with PBS. DMEM supplemented with 1% I.T.S followed by the addition of Mstn, Fstn, Activin A or control. Cells were maintained in treatment for 48 hour then harvested in TRIZOL® reagent (Invitrogen) for RNA isolation and subsequent cDNA synthesis (Bio-Rad). 50-100 ng of cDNA was amplified in duplicates in each 40-cycle reaction using an iCycler™ (Bio-Rad) with annealing temperature set at 60⁰C, ABsolute™ QPCR SYBR® Green Fluorescein Mix (ABgene, Thermo Fisher Scientific), and custom-designed qRT-PCR primers (Table 1). A melt curve was used to assess the purity of amplification products. mRNA levels were normalized to β-Actin/18S and gene expression was calculated as fold change using the comparative CT method. If not otherwise indicated, treated groups were compared to PBS control groups.

ANOVAs showed significant treatment effects for activin A, myostatin, and follistatin treatment of primary myoblasts (Fig. 7A). Activin A produced a significant dose-response increase in proliferation in myoblasts from younger mice but not older mice; however, the treatment*age interaction was not significant. Follistatin also increased proliferation in dose-response manner in young myoblasts, but the effect was attenuated in myoblasts from older mice (Fig. 7B). There was a significant ($p=.001$) treatment*age interaction effect for follistatin treatment where the treatment effect was much greater in younger myoblasts compared to older cells. Myostatin treatment also increased proliferation in young myoblasts compared to untreated cells, whereas in older myoblasts myostatin decreased proliferation at the low dose (Fig. 7C). There was also a significant ($p<.001$) treatment*age interaction effect for myostatin, where myostatin treatment increased proliferation in young myoblasts but decreased proliferation in the older cells.
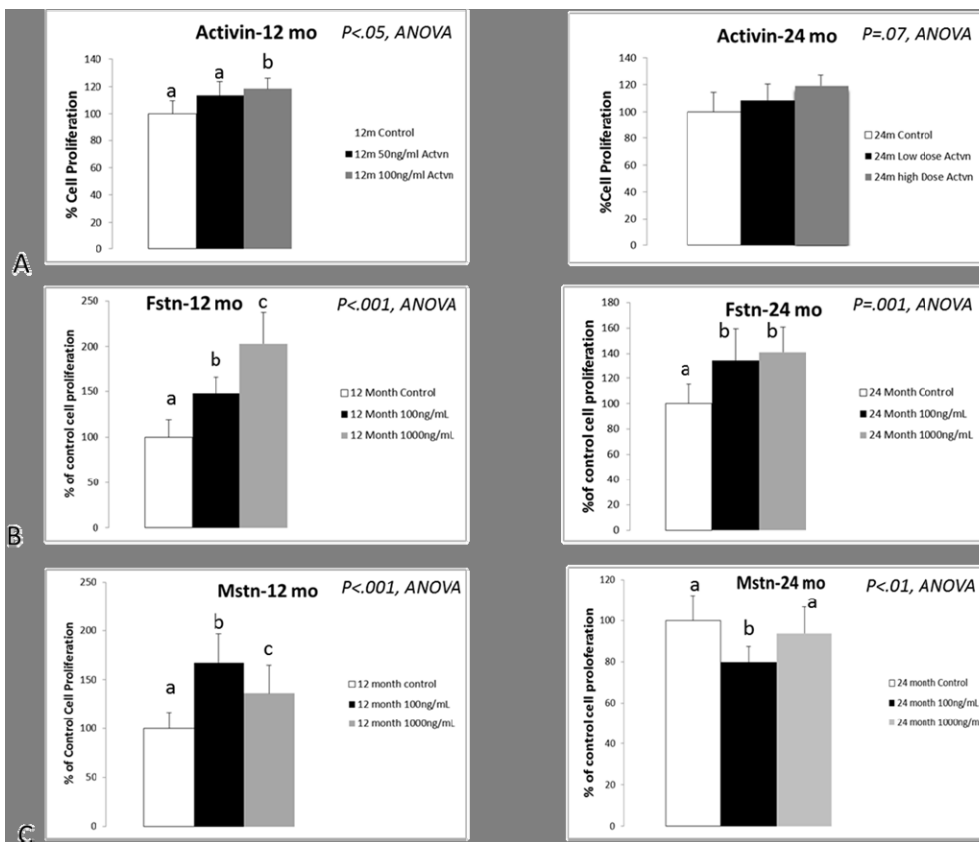
Figure 7. *Proliferation assays of primary myoblasts isolated from young (12 mo, left column) and aged (24 mo, right column) mice treated with activin A (A), follistatin (B), or myostatin (C).*

RT-PCR data revealed no marked changes in the expression of differentiation markers MyoD, myogenin, or myosin heavy chain (MHC) with Activin A treatment.   MyoD and MHC expression were also unaffected by follistatin treatment of young and aged myoblasts; however, follistatin stimulated a significant increase in myogenin expression in aged but not young myoblasts (Fig. 8A). ANOVAs demonstrated a significant age effect for myogenin expression ($p<.05$), and there was also a significant treatment * age interaction for myogenin expression. Myostatin treatment had no effect on MyoD or myogenin expression in either young or aged myoblasts, but myostatin did produce a significant increase in MHC expression in aged but not young myoblasts (Fig. 8B).
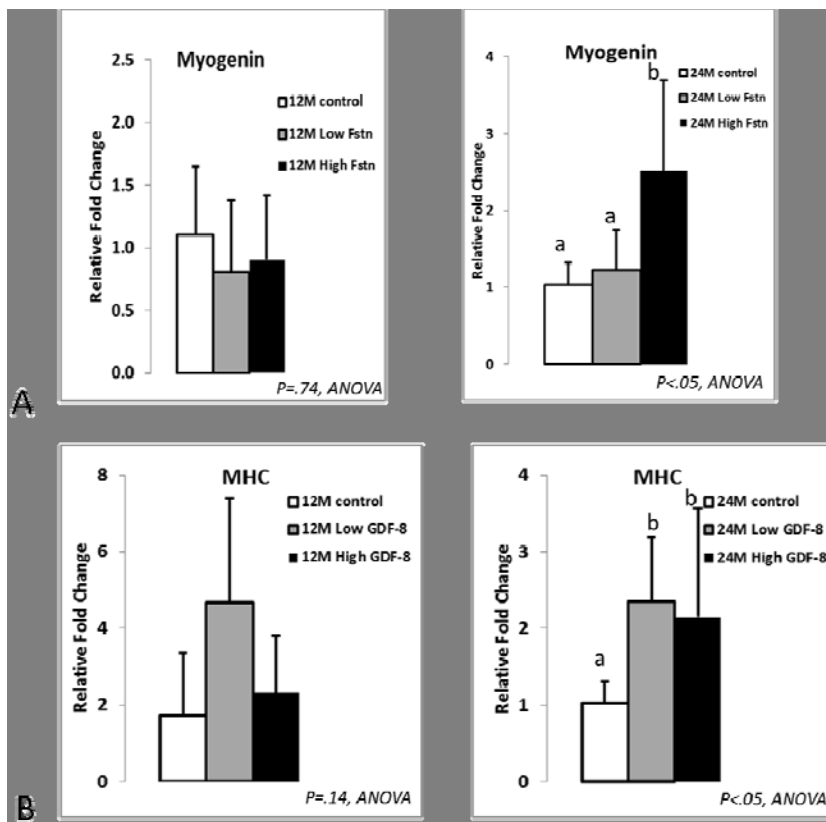
Figure 8*. Gene expression of the differentiation marker myogenin (top) and myosin heavy chain (MHC) in young (12M, left column) and aged (24M, right column) primary myoblasts treated with follistatin (A) and myostatin (B; GDF-8).*

Primary BMSC experiments for Aim 2, task 2: In Aim 1 we found that activin A was also elevated with age in bone marrow, and so we extended our treatments in aim 2 to include activin A, myostatin, and follistatin. Bone marrow aspirates were flushed from femora and magnetic nanoparticles conjugated to anti-mouse CD11b, CD45R/B220, and Pan DC monoclonal antibodies were used to remove hematopoietic-lineage cells. A round of positive-selection was then performed using anti-Sca-1 microbeads. Enriched BMSCs were cultured in proliferation medium (DMEM supplemented with 10% heat-inactivated FBS) in T-75 flasks until ~80% confluent. Cells were then lifted with trypsin/EDTA, plated in 96-well plates at a density of 5,000 cells/well in proliferation medium, and allowed to attach for 24 h. Proliferation medium was removed, cells washed with PBS, and DMEM supplemented with with 2% heat-inactivated FBS (for BMSCs) was added followed by control (PBS), activin A, follistatin, or myostatin (all from R&D Systems, Minneapolis) at the same doses noted above for primary myoblasts. After 24 h of treatment, MTS reagent was added according to the manufacturer's protocol (Promega, Madison, WI) and absorbance at 492 nm was read 2 h later. Osteogenic differentiation and Alizarin Red S (ARS) staining was performed as described previously (Zhang et al., 2008)

ANOVAs showed a significant (P<.001) age effect in each treatment group, with younger BMSCs overall having higher values for proliferation than older BMSCs, irrespective of the treatment (Fig. 9). Proliferation assays showed no effects of activin treatment on either young or old BMSCs (Fig. 9A). Two-factor ANOVA revealed a significant treatment*age interaction for follistatin, with follistatin having no impact on proliferation in older BMSCs but reducing proliferation in young BMSCs (Fig. 9B). Myostatin significantly decreased BMSC proliferation in both age groups (Fig. 9C); however, this effect was greater in the aged cells, and this treatment*age interaction was also significant (P<.05) for myostatin.
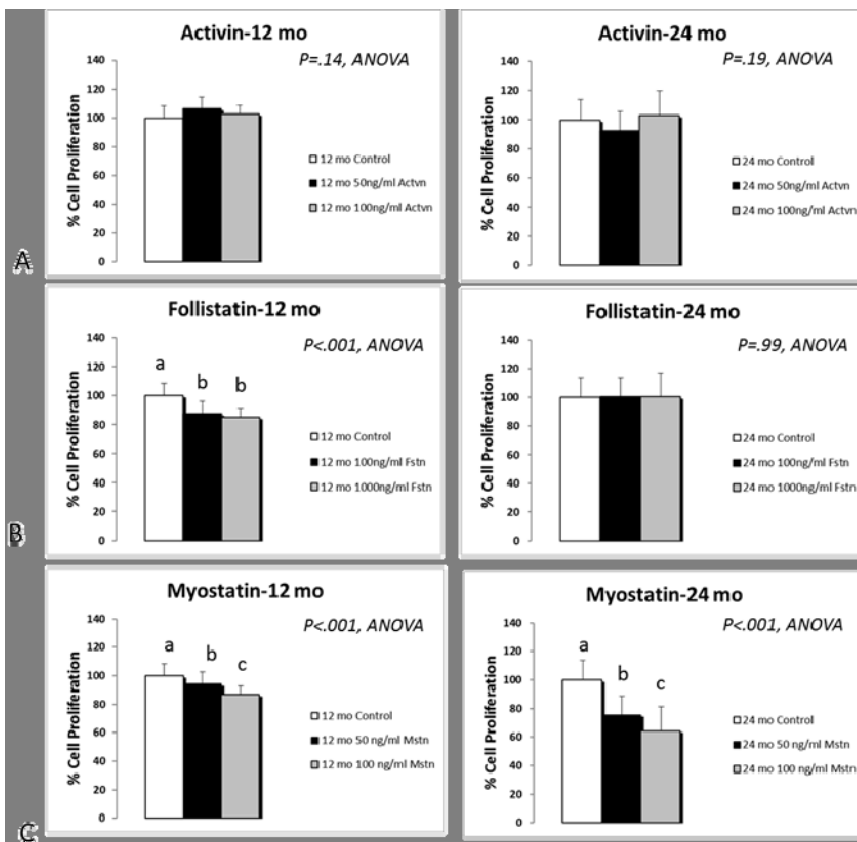
Figure 9. *Results of proliferation assays following treatment of primary bone marrow stromal cells (BMSCs) with activin A (A; activin), follistatin (B; Fstn), and myostatin (C; Mstn). Means with different superscripts differ significantly from one another (P<.05).*

Differentiation assays using alizarin red staining to detect mineralization revealed that activin treatment significantly increased mineralization of young and older BMSCs (Fig. 10). The effect of age on mineralization was significant (P<.001) for activin, with the younger cells consistently showing greater mineralization in response to treatment. Treatment effects were less pronounced in aged BMSCs, with the low dose of activin increasing alizarin red staining but the other doses showing no significant effect (Fig. 10C, D). The treatment*age interaction was significant (P<.01) for activin using two-factor ANOVA. In the myostatin experiments, the higher dose of myostatin significantly decreased mineralization in younger cells but not in older cells, and the treatment*age interaction was also significant for myostatin (P<.01). Finally, follistatin treatment did not significantly affect mineralization either young or older BMSCs (Fig. 10).
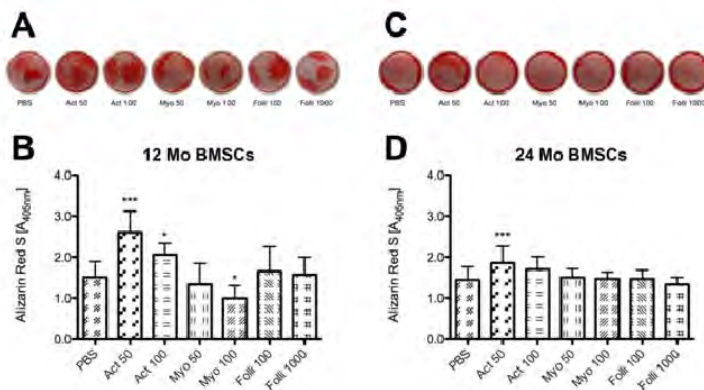

Figure 10. *Alizarin red staining of bone marrow stromal cells cultured in osteogenic conditions. Images of wells (top row) and quantification of staining (bottom row) in BMSCs from young mice (A, B; 12 Mo BMSCs) and older mice (C, D; 24 Mo BMSCs) treated with Activin A (Act), Myostatin (Myo) or Follistatin (Folli) at 50 ng/ml (50), 100 ng/ml (100) or 1000 ng/ml (1000). ***P<.001, *P<.05 relative to same-aged PBS controls.*

Task 2 status: Completed. There are two myostatin inhibitors that can be used, one that is myostatin-specific (propeptide) and one that binds both myostatin and activin A (decoy receptor). Prior to determining which is optimal we needed, in aim 2, to determine the effects of

myostatin and activin on muscle and bone cells. Our data indicate that activin A can have positive effects on myoblast proliferation, and activin also enhances mineralization. Thus, a decoy receptor is not an optimal inhibitor because of its effect of blocking activin activity, hence a myostatin-specific inhibitor (propeptide) the optimal therapeutic to be validated in aim 3.

Milestone 2: The optimal treatment and dose of myostatin inhibitor for enhancing osteogenesis and myogenesis in aged BMSCs and myoblasts will have been determined in vitro. Our data in aim 2 indicate that the myostatin propeptide is the optimal inhibitor because of the contrasting effects of myostatin vs activin A in vitro.

Aim 3 (months 24-36). Determine the effects of myostatin inhibitors on muscle and bone aging in vivo.

Task 1 (months 24-30). Mice 22 months of age will be treated with a myostatin propeptide. Effects on behavioral performance and measures of bone and muscle anabolism will be determined.

Animals & treatments: C57BL6 mice were purchased from the aged rodent colony at the National Institute on Aging, National Institutes of Health (USA) at 22 months of age and delivered to Georgia Regents University, Augusta GA. Animals were allowed to acclimate for one week and were maintained at the Laboratory Animal Service Facility of Georgia Regents University. An earlier dose-response study was used to evaluate the efficacy of a myostatin propeptide in vivo (Hamrick et al., 2010). Adult mice (5-6 mo.) were treated with the propeptide at 0, 10, 20, or 50 mg/kg at day 0, 5, and 10 and then sacrificed one week after the last treatment. Those data showed that propeptide treatment increased fore- and hindlimb muscle mass by 10% at the 10 mg/kg dose and increased muscle mass by more than 15% at the 20 mg/kg dose, but the 50 mg/kg dose did not increase muscle mass beyond the increase observed in the 20 mg/kg group (Hamrick et al., 2010). The 20 mg/kg dose was therefore used in this study. Mice were divided into two treatment groups: a vehicle group (VEH; n=14) and a myostatin propeptide group (PRO; n=15). Mice received i.p. injections every five days for 25 days with a dosage of 20 mg/kg body weight at a volume of 0.2 ml. We used a four- rather than eight-week treatment period because the animals were old enough we were concerned they might die of natural causes prior to completion of the study. Myostatin propeptide [4.48mg/ml] was obtained from Pfizer Inc (Cambridge, MA, USA). Mice were given calcein i.p. injections to label actively mineralizing bone surfaces four days and 24 hours prior to sacrifice.

Myostatin propeptide increases muscle mass and fiber size in aged mice: Body weight of the vehicle- and propeptide-treated animals was similar at the end of the study (Fig 11A). Each treatment group did, however, lose some weight over the treatment period but this was less dramatic for the treated animals, such that their decrease in body weight from day 0 to day 25 was significantly less than that of the vehicle-treated mice (Fig. 11B).
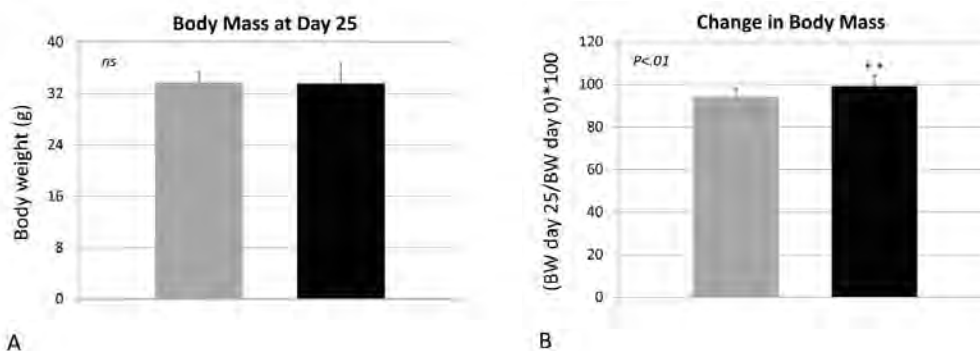

Figure 11. *Body mass (A) and change in body weight (B) for animals treated with either saline (VEH) or myostatin propeptide (PRO) weekly for over four weeks.*

Muscle mass of the tibialis anterior was significantly increased in the treated mice, both absolutely (Fig. 12A) and relative to body weight (Fig. 12B). Fiber size of the predominantly fast-twitch extensor digitorum longus (EDL) muscle was also significantly increased by more than 15% in the treated mice (Fig. 12C), whereas the increase in muscle fiber size in the predominantly slow-twitch soleus (SOL) muscle was also increased significantly (P<.05) but by a lesser magnitude (~5%).
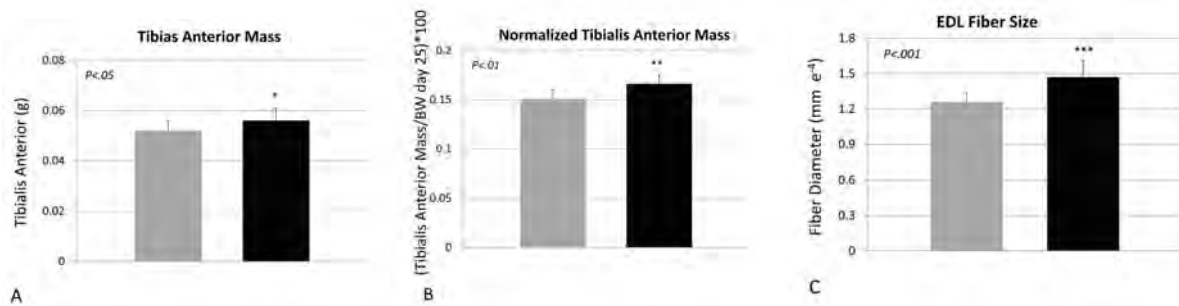

Figure 12. *Muscle parameters for mice treated with saline (VEH) or myostatin propeptide (PRO) weekly for a period of four weeks. (A) Tibialis anterior mass, (B) tibialis anterior mass relative to body weight, (C) extensor digitorum longus fiber diameter (EDL).*

Propeptide treatment produced a slight but non-significant increase in the expression of myostatin itself, as well as expression of myosin heavy chain and IGF-1 (Fig. 13). Surprisingly, expression of the ubiquitin ligases Murf1 and Mafbx was significantly increased with propeptide treatment (Fig. 13), and the PCR data were further validated by Western blot (Fig. 13).
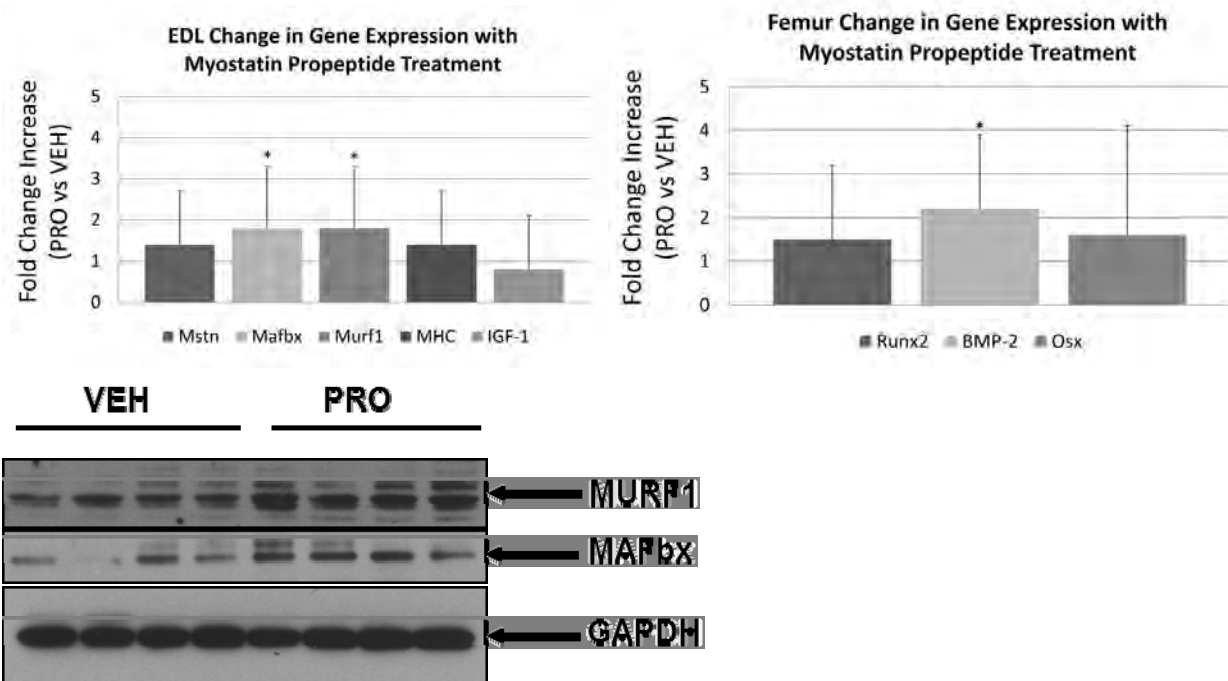
Figure 13. *Real-time PCR data (top row) for mice treated with saline (VEH) or myostatin propeptide (PRO) weekly for a period of four weeks showing increased expression of Murf1 and Mafbx in PRO-treated mice (left), and increased expression of BMP-2 in mice treated with propeptide (right). *P<.05. Western blot data from muscle (bottom) showing increased Murf1 and Mafbx in treated muscle.*

Myostatin inhibitor significantly increases grip strength in aged mice. We assessed musculoskeletal performance in vehicle (control) and propeptide (pro) treated mice using behavioral outcome measures in Small Animal Behavior Core (SABC) at Georgia Regents University. Rotarod tests, measures of free cage activity, and grip strength tests were performed over a period of approximately 5 days both before and after the four-week treatment

period. Results demonstrate that rotarod performance was similar for control and treated groups both before and after treatment, however grip strength was significantly increased in the treated mice (Figure 14).
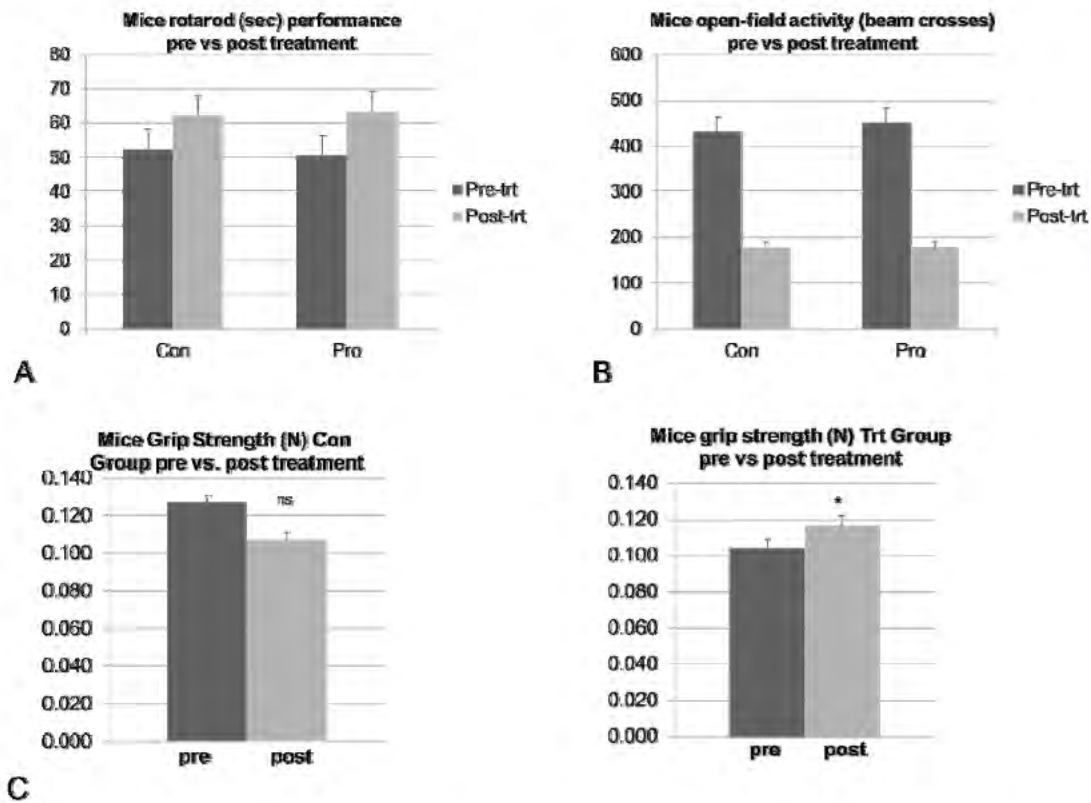

Figure 14. Behavioral performance data for mice treated with saline (Con) or myostatin propeptide (Pro) both per- and post-treatment showing similar measures for rotarod (A) and open-field activity (B), but increased grip strength (C) in the propeptide treated mice. *P<.05.

Myostatin inhibitor does not alter bone formation or bone strength in aged mice: MicroCT data from the tibia show that bone mineral density is actually slightly higher (3%) in the tibias of vehicle-treated mice (Table 1), but other parameters such as bone volume relative to total volume, trabecular number, and trabecular thickness are similar between the two groups (Table 1). Likewise, three-point bending tests of tibias show that ultimate force, stiffness, and toughness (energy to fracture) are also similar between the vehicle- and propeptide-treated mice (Table 1).

Table 1. microCT and biomechanical testing of the proximal tibia for mice treated with saline (VEH) or myostatin propeptide (PRO; 20 mg/kg). BMD=bone mineral density, BV/TV=bone volume relative to total volume, Tb.Th=trabecular thickness, Tb.N=trabecular number, Fu=ultimate force, U=energy-to-fracture, S=stiffness.

Parameter	VEH (n=14)	PRO (n=15)	p value
BMD	1.43±0.06	1.38±0.05	.01
BV/TV	6.67±2.37	6.14±2.16	.24
Tb. Th	0.11±0.02	0.11±0.01	.47
Tb. N	0.59±0.14	0.54±0.16	.23
Fu (kg)	2.21±.40	2.18±.34	.39
U (kg/um^2)	740.6±417.5	670.3±309	.31
S (g/um)	4.6±2.0	4.7±2.0	.44

Bone histomorphometry data reveal that osteoblast and osteoclast numbers do not differ between the experimental groups (Table 2). Fluorochrome labeling showed double-labels in only three mice from each group, and so single-labeled surfaces were compared. Actively mineralizing surfaces were also similar between the two groups of mice (Table 2).

Table 2. Bone histomorphometry data for the distal femur of mice treated with saline (VEH) or myostatin propeptide (PRO; 20 mg/kg).N.Ob/BS=osteoblast number per bone surface, MS/BS=mineralizing surface (single-label) relative to bone surface, N.Oc/BS=osteoclast number per bone surface.

Parameter	VEH (n=15)	PRO (n=14)	p value
N.Ob/BS	27.26±17.49	25.09±9.31	.14
MS/BS	0.41±0.17	0.43±0.13	.34
N.Oc/BS	6.33±2.61	6.18±3.82	.38

Gene expression data show no significant differences in the expression of osteogenic genes Osx or Runx2 with propeptide treatment, however the expression of BMP-2 is increased in animals receiving the propeptide (Fig. 13).

Our data show that PRO treatment significantly increases muscle fiber size and muscle mass, both absolutely and relative to body weight. In contrast bone volume, bone strength, and histomorphometric parameters of bone formation and bone resorption were unchanged with PRO treatment. Our findings are consistent with previous studies utilizing a myostatin antibody in aged mice showing that targeting myostatin increases muscle fiber size and mass; however, our data differ from work recently published by Chiu et al. (2013, *J. Gerontol. A Biol. Sci. Med. Sci*) utilizing a decoy myostatin receptor (ActRIIB-Fc) showing that ActRIIB-Fc appears particularly effective at increasing bone density and bone formation. The anabolic effects of ActRIIB-Fc on aged bone are likely due to the ability of this molecule to antagonize other ligands besides myostatin, such as activin or bone morphogenetic proteins. There are several additional points to consider though when comparing the effects of the two inhibitors. If increases in muscle mass with inhibitor treatment might prevent or delay, not necessarily reverse, bone loss then a myostatin propeptide or antibody could be a safe and effective prophylactic approach for age-related bone loss. Perhaps more importantly, as noted below, the decoy receptor does not appear to be safe.

Task 1 status: The overall goals of task 1 were completed, and we were able to complete the grip strength and rotarod studies with the cost-extension into FY14; however, although the grip strength measures showed a significant change with treatment, the other behavioral measures were quite similar. We have developed an IACUC-approved video system for analyzing gait mechanics which we believe will have greater resolution than the rotarod and ambulatory tests, and we proposed to apply this technology in the extension period for FY15. In addition, our serum assays showed significantly elevated IL-6, an inflammatory cytokine, in propeptide-treated mice. This cytokine is regulated by several important microRNAs (miRNAs) of the let7 family in skeletal muscle, and we are seeking an extension into FY15 to analyse these miRNAs.

Task 2 (months 30-36). Mice 22 months of age will be treated with a soluble decoy myostatin receptor for 8 weeks. Effects on behavioral performance and measures of bone and muscle anabolism will be determined.

Task 2 status. Since the CDMRP application was submitted, Acceleron decided to cease their Phase II trial of the soluble decoy myostatin receptor (ACE-03) because of safety issues (http://quest.mda.org/news/ace-031-clinical-trials-duchenne-md-stopped-now). Acceleron then announced that they will no longer pursue ACE-031 (http://www.acceleronpharma.com/2013/05/acceleron-and-shire-conclude-collaboration-on-ace-031/) this drug because of the safety issues. We therefore decided not to pursue task 2, since

the molecule now appears to be unsafe in humans, and because our data in aim 2 indicate that activin A can have positive effects on myoblast proliferation, and activin also enhances mineralization. Thus, a decoy receptor is not an optimal inhibitor because of its effect of blocking activin activity

Milestone 3: The optimal myostatin inhibitor for enhancing osteogenesis and myogenesis in aged rodents will be determined in vivo. The data presented in aim 3, when taken in consideration of the Acceleron trials noted above, suggest that a myostatin propeptide is likely to be a safe and effective molecule for enhancing muscle mass with aging.

Year 4 Addendum (May, 2014-November, 2014):

We have identified certain small molecules (miRNAs) as markers of muscle anabolism, and we requested additional time to analyze and validate these in samples we have already collected, since the start of our previous extension was delayed by several months. In addition, our first round of rotarod and activity measures were somewhat inconclusive. We therefore developed an IACUC-approved video system for analyzing gait mechanics which we believe will be more useful and we propose to apply this technology in the extension period. These objectives are all included within the original statement of work. Results from the extension period using MaxTraq software and high-speed video of mice on a motorized treadmill indicate that gait parameters such as stride length and contact time are similar between treated and control (vehicle) mice (Fig. 15a). Our miRNA analyses indicate that propeptide treatment significantly alters the expression of multiple microRNAs in the muscle of aged mice (Fig. 15b). This finding points to a novel mechanism of myostatin inhibition on muscle mass. Specifically, previous studies have focused on a role for myostatin in altering IGF1-mTor signaling or the abundance of degradative proteins termed ubiquitin ligases (Arounleut et al., 2013). The data shown in Fig. 15a suggest that myostatin propeptide may stimulate myogenesis by upregulating miR-23b, as miR-23b is known as a myogenic miRNA (Dmitriev et al., 2013). The myostatin propeptide also upregulates let-7e, and the let-7 group of miRNAs are known to suppress cell proliferation. Myostatin propeptide may therefore favor myogenic differentiation over cell proliferation in aged skeletal muscle, thereby increasing muscle mass and strength.
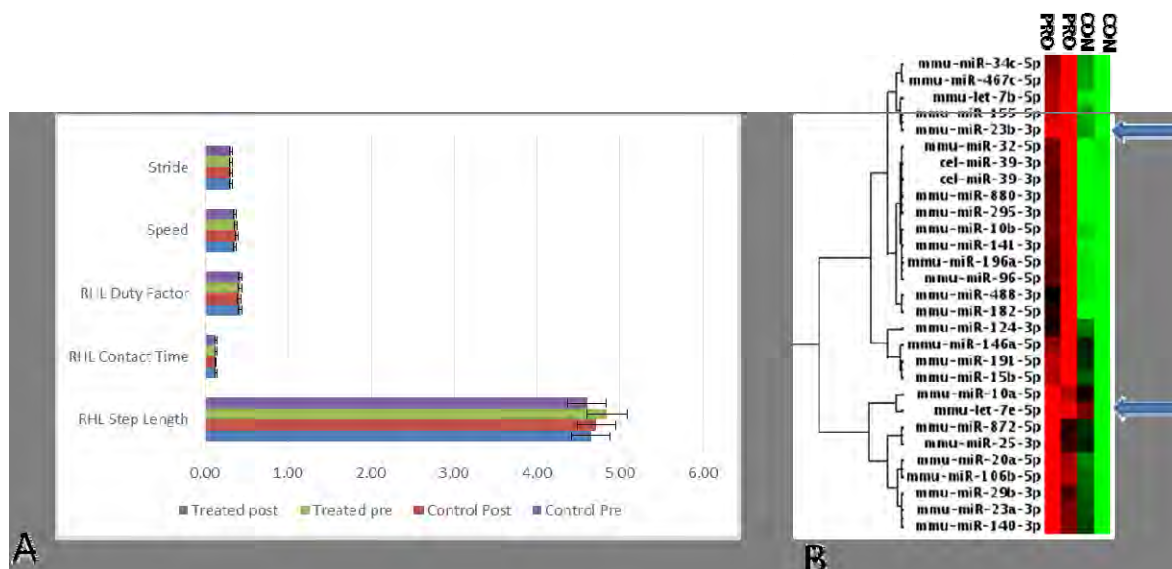
Figure 15. *A. Quantitative gait parameters for treated (propeptide) and control (saline) aged mice before (pre) and after (post) the four week treatment period. Measures for stride time (sec), speed (sec) and right hind limb (RHL) step length (cm) are similar between groups. Error bars represent +/- 5%. B. PCR array data showing increased (red) expression of miR-23b and let-7e (blue arrows) in muscles from mice with propeptide treatment.*

Key Research Accomplishments in Year 4:

- We published (*Experimental Gerontology*) the first study on the effects of myostatin propeptide in aged rodents, and featured our research on myostatin in *Physiology*.
- The propeptide treatment data were presented in the form of plenary podium presentations at the 2013 meeting of the American Society for Bone & Mineral Research in Baltimore, MD; the 2014 meeting of the International Bone & Mineral Society in Sun Valley, Idaho; and the 2014 meeting of the American Society of Nephrology in Philadelphia, Pennsylvania.
- Our findings were also presented in the form of invited keynote addresses as the Fesler-Lampert Chair in Aging Studies and the Center on Aging, University of Minnesota, Minneapolis, Minnesota, and the keynote speaker for the Driskill College of Graduate Studies, Northwestern University, Chicago, Illinois.

Reportable Outcomes:

Manuscripts:

2015   Novotny S, Warren G, **Hamrick MW**. Aging and the muscle-bone relationship. *Physiology.* 30: 8-16.

2013   Arounleut P, Bialek P, Liang L, Upadhyay S, Fulzele S, Johnson M, Elsalanty M, Isales CM, **Hamrick MW**. A Myostatin Inhibitor (Propeptide-Fc) Increases Muscle Mass and Muscle Fiber Size in Aged Mice but Does not Increase Bone Density or Bone Strength. *Experimental Gerontology* 48:898-904.

2013   Bowser M, Chutkan N, Martell J, Corpe S, Park MA, Hillman D, Ahsan S, Arounleut P, Isales CM, Shi XM, **Hamrick MW** . Age-related changes in the activin A-myostatin-follistatin system within the bone marrow microenvironment. *Experimental Gerontology* 48: 290-97.

2013   Elkasrawy M, **Hamrick MW**. Myostatin (GDF-8) signaling in progenitor cells and applications to bone repair. In *Stem Cells & Bone Tissue* (R. Rajendram, V. Preedy, V. Patel, eds.), Ch. 8, pp. 145-160. CRC Press: London.

2012   **Hamrick MW**. The skeletal muscle secretome: an emerging player in muscle-bone crosstalk. *Nature Bonekey* 1: 60.

2012   Elkasrawy M, Fulzele S, Bowser M, Wenger K, **Hamrick MW**. Myostatin (GDF-8) inhibits chondrogenesis and chondrocyte proliferation in vitro by suppressing Sox-9 expression. *Growth Factors* 29:253-262.

2011   **Hamrick, MW**. A role for myokines in muscle-bone interactions. *Exercise & Sports Science Reviews* 39: 43-47.

Abstracts from Professional Presentations:

2014   Periyasamy-Thandavan S, Herberg S, Arounleut P, Upadhyay S, Kondrikova G, Dukes A, Davis C, Johnson M, Shi X, Isales CM, **Hamrick MW**, Hill WD. The Age-Associated Rise in miRNAs from Muscle Target SDF-1 and Musculoskeletal

Regulatory Genes is Reversed with Caloric Restriction and Leptin. J Bone Miner Res FR0194.

2014   Herberg S, Periyasamy-Thandavan S, Arounleut P, Upadhyay S, Dukes A, Davis C, Kondrikova G, Johnson M, Isales CM, Hill WD, **Hamrick MW**. Mediation of SDF-1/CXCR4 signaling in aged skeletal muscle by the adipokine leptin. J Bone Miner Res FR0197.

2014   Rabey K, McNeill J, Wu C, Bordas J, Guilak F, **Hamrick MW**, Schmitt D. Effects of aging and nutrition on bone microstructure and gait. Am J Phys Anthropol 31: 214.

2013   Arounleut P, Bialek P, Elsalanty M, Upadhyay S, Isales CM, Hill WD, Shi X, **Hamrick MW**. A Myostatin Inhibitor (propeptide-Fc) Increases Muscle Mass but Does Not Alter Bone Density or Strength in Aged Mice. J Bone Miner Res Supplement: 1011.

2012   Shi X, Bowser M, Yang N, He L, Herberg S, Fulzele S, Hill WD, Isales CM, Hamrick MW. Effects of Activin A and Follistatin on the Differentiation of Aged Primary Bone Marrow Stromal Cells (BMSCs) and Primary Myoblasts in vitro. J Bone Miner Res Supplement: SU 0179.

2012   Bowser M, Fulzele S, Ahsan S, Arounleut P, Isales CM, **Hamrick MW**. Changes in the activin A-myostatin-follistatin system within bone and muscle of aging mice. FASEB J 26: 914.4.

2012   Elkasrawy M, Fulzele S, Hill W, Isales CM, **Hamrick MW**. Myostatin (GDF-8) suppresses Wnt/β-catenin signaling during chondrogenesis in vitro. The Hilton Head Workshop on Regenerative Medicine.

2011   Bowser M, Chutkan N, Martell J, Corpe R, Isales CM, Park MA, Hillman D, **Hamrick MW**. Age-related changes in the activin A-myostatin-follistatin system within the bone marrow microenvironment.   Journal of Bone & Mineral Research SA0001.

2011   Zhang W, **Hamrick MW**, Ding K, Wenger K, Hill W, Isales CM, Shi XM. Bone marrow mesenchymal stem cell and bone loss with aging. ASBMR Forum on Aging & Skeletal Health 29: P8.

2011   Isales CM, **Hamrick MW**, Ding K, Zhong Q, Bollag W, Shi XM, Hill W, Rowse J, Elsalanty M, Chutkan N, Insogna K. The impact of dietary protein on bone mass and strength in the aging animal. ASBMR Forum on Aging & Skeletal Health 34: P17.

2011   Elkasrawy M, Immel D, Wen X, Liu L, Lian L, **Hamrick MW**. Effects of myostatin on muscle and bone healing following deep penetrant musculoskeletal injury. British Journal of Bone & Joint Science: P053.

Invited Seminars:

2014   Keynote address, Driskill Graduate Program in the Life Sciences, Feinberg School of Medicine, Northwestern University, Chicago, Illinois.

2014   Symposium on The Muscle-Bone Unit in Chronic Kidney Disease, American Society of Nephrology annual meeting, Philadelphia, Pennsylvania.

| 2014 | Symposium on Skeletal Muscle and Bone Biomechanical Properties, International Bone & Mineral Society annual meeting, Sun Valley, Idaho. |

| 2013 | The Fesler-Lampert Chair in Aging Studies and the Center on Aging, University of Minnesota, Minneapolis, Minnesota. |

| 2013 | College of Health Sciences, University of Delaware, Newark, Delaware. |

| 2013 | Symposium on Muscle & Mobility, Abbott Nutrition, Columbus, Ohio. |

| 2013 | Progress in Bone Biology: Ageing & the Skeleton, Bone Academy, Vienna, Austria. |

| 2013 | International Webinar, International Bone & Mineral Society, New Directions in Muscle-Bone Interactions. |

| 2012 | The Hilton Head Workshop on Regenerative Medicine, Hilton Head, SC. |

| 2012 | Symposium on Muscle-Bone Crosstalk, American Physiological Society, Experimental Biology, San Diego, California. |

| 2011 | Plenary Symposium on Muscle-Bone Interactions, American Society for Bone & Mineral Research, San Diego, California. |

| 2011 | Augusta Research Symposium on Advances in Warrior Care, Augusta, GA. |

| 2011 | Program in Musculoskeletal Research, Eli Lilly & Co., Indianapolis, Indiana. |

| 2011 | Department of Pathology & Anatomical Sciences, University of Missouri, Columbia MO. |

Conclusions:

Falls and debilitating bone fractures are a major problem for veterans, and more than 40,000 veterans suffered hip fractures from 2000-2002. Men have a higher fracture-related mortality than women, and one out of every three male veterans that sustains a hip fracture dies within one year. Falls are the main etiological factor in more than 90% of fractures, and so treatments that can improve muscle strength while at the same time increasing bone mass will significantly reduce fracture-related morbidity and mortality. Myostatin is a factor that induces muscle wasting and suppresses bone formation. Our data collected thus far demonstrate i) myostatin suppresses proliferation in aged, but not young, myoblasts, ii) myostatin is elevated with age in muscles composed primarily of slow-twitch fibers (e.g. soleus), iii) myostatin propeptide (inhibitor) increases muscle mass and muscle fiber size in aged mice, but does not alter bone density or bone strength, and iv) myostatin propeptide increases the expression of microRNAs (e.g., miR-23b) in aged mice that are associated with myogenesis. These findings suggest that myostatin inhibitors may have potential for suppressing muscle wasting and improving muscle repair in older individuals, but their effect on bone may be less significant.

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Arounleut P, Bialek P, Liang L, Upadhyay S, Fulzele S, Johnson M, Elsalanty M, Isales CM, Hamrick MW. (2013) A Myostatin Inhibitor (Propeptide-Fc) Increases Muscle Mass and Muscle Fiber Size in Aged Mice but Does not Increase Bone Density or Bone Strength. *Experimental Gerontology* 48:898-904.

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Appendices: Articles recently published.

Aging and the Muscle-Bone Relationship

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Aging-induced declines in muscle size and quality are thought to contribute to catabolic alterations in bone, but changes in bone with age also profoundly alter its response to muscle-derived stimuli. This review provides an overview of some of the alterations that occur in muscle and bone with aging, and discusses the cellular and molecular mechanisms that may impact these age-associated changes.

Over the next 10 years, the number of people in the world over the age of 65 is projected to increase on a percentage basis at a rate almost four times faster than that for the younger population (7). As the size of the older population increases, so does the occurence of aging-related morbidities such as osteoporosis and sarcopenia. Bone fractures are directly linked to osteoporosis in aging adults, and in the U.S. the annual incidence of bone fractures in women exceeds the annual incidence of stroke, breast cancer, and heart disease combined (62). Hip fractures in particular are associated with significant morbidity and mortality, and of those suffering a hip fracture, roughly 40% will require nursing home care, and 20% will not walk again (24). Aging is not only associated with a loss of bone density and strength but is also associated with a reduction in muscle mass and strength referred to as sarcopenia. A consensus definition of sarcopenia has not been reached; however, most assessments involve declines in muscle functional capacity (e.g., strength) (11) and/or a morphometric measure (e.g., cross-sectional area, muscle mass). The muscle weakness that occurs with sarcopenia increases the risk for falling (38), which further increases the propensity for bone fracture.

Strong associations between muscle and bone size have been reported across the lifespan (16, 57). Since the late 1800s, it has been assumed that a causal relationship exists between muscle and bone and that this allometric scaling relationship is mechanical in nature (80–82). That is, muscle and bone are proportionally matched in their functional capacity and geometric structure. This relationship does, however, appear to change significantly with age. For example, the capacity for muscle to generate force declines with age, and the anabolic reponse of bone to muscle-derived stimuli also appears to be altered with age. In addition, muscle is now recognized to have paracrine and endocrine effects that may also influence bone independent of a mechanical relationship (26, 27). Here, we review some of the basic mechanisms by which muscle and bone are thought to interact throughout life, and how these may change with age, leading to bone loss and bone fractures. In *The Mechanical Relationship Between Muscle and Bone*, we discuss the mechanical relationship between muscle and bone, with particular emphasis on the role of muscle in affecting bone size and bone geometry. In *Impact of Aging on the Muscle-Bone Relationship*, we assess whether the muscle-bone relationship remains relatively constant with aging in both animals and humans. *Cellular and Molecular Mechanisms Underlying Age-Related Changes in the Muscle-Bone Relationship* addresses the cellular and molecular mechanisms linking bone and muscle that are altered with age and which negatively impact cross talk between the two tissues.

The Mechanical Relationship Between Muscle and Bone

The musculoskeletal system provides the mechanical framework for movement that is powered by contractions of skeletal muscles. To overcome the anatomical disadvantage associated with short lever arms, many muscles are required to produce high forces to generate movement. The muscle-generated loads applied to bone can far exceed external loads resulting from the body interacting with its environment (e.g., ground reaction forces) (19, 35, 46, 47, 54). Specifically, the utilization of instrumented proximal femoral prostheses have demonstrated that the forces produced during muscle contraction can account for >70% of the bending moments applied to the lower limb (46). Bone size and mechanical properties are, therefore, thought to allometrically scale to the magnitude of peak muscle forces (65) in response to mechanical loading (e.g., through increased or decreased use). Julius Wolff ("Wolff's Law") was the first to describe that bone changes its external shape and internal structure in response to mechanical loads imposed on the skeleton (80–82).

The inclusion of muscle as the primary source of mechanical loading was a central principle of the Utah paradigm of skeletal physiology and the mechanostat hypothesis (21, 22). Specifically,

healthy postnatal bone was proposed to adapt its structure and strength to the typical peak mechanical loads it experiences, which are applied via muscular contractions during exercise and/or a person's activities of daily living (13, 21, 41, 45). Bone's detection and transmission of mechanical loading stimuli by its resident cell populations is referred to as mechanotransduction, where the stimulus is the result of bone strain (i.e., the change in length per unit of original length) (4, 12, 15, 25, 42, 79). According to the mechanostat hypothesis, strain magnitudes experienced by mechanosensing cells in the tissue are compared against threshold values to determine whether feedback mechanisms and adaptive responses are necessary. Strains above 3,000 microstrain ($\mu\epsilon$) typically trigger bone formation, whereas strains of <500 $\mu\epsilon$ trigger bone resorption. The dense network of osteocytes trapped within the mineralized bone matrix is the primary sensor of changes in bone loading, which occurs primarily through changes in fluid movement within the lacuno-canalicular system of bone. These changes in fluid movement in turn stimulate many downstream effects, including Wnt/beta catenin signaling that promotes the downregulation of sclerostin, a potent inhibitor of bone formation (5, 84). Muscle weakness or muscle atrophy is associated with bone loss, even in the absence of changes in load bearing. Specifically, muscle paralysis using botulinum toxin (botox) is observed to decrease bone mass even during hindlimb unloading, where the effects of paralysis on load bearing are removed (75). The mechanical communication between muscle and bone allows for anabolic/catabolic modifications to bone in response to loading that apparently attempt to maintain a constant relationship between the functional and structural capacities of the two tissues.

The long bones of the appendicular (limb) skeleton and shorter bones of the axial (spine) skeleton have a dense outer cortex and a spongy network of trabecular bone filling the vertebrae and the articular ends of long bones. Approximately 80% of the total mass of the skeleton is cortical bone, and, with aging, 70% of all bone lost is cortical (69). The nonvertebral (limb) skeleton is primarily cortical bone, and, of all fractures that occur, 80% are nonvertebral (69). Thus a key strategy for preventing bone fractures is to either prevent loss of cortical bone with aging or increase cortical bone formation with aging. The bone cortex has an outer periosteal layer that deposits bone peripherally, which ultimately increases the bone's external diameter, overall cross-sectional area, and resistance to bending loads (FIGURE 1, A AND B). Notably, bone
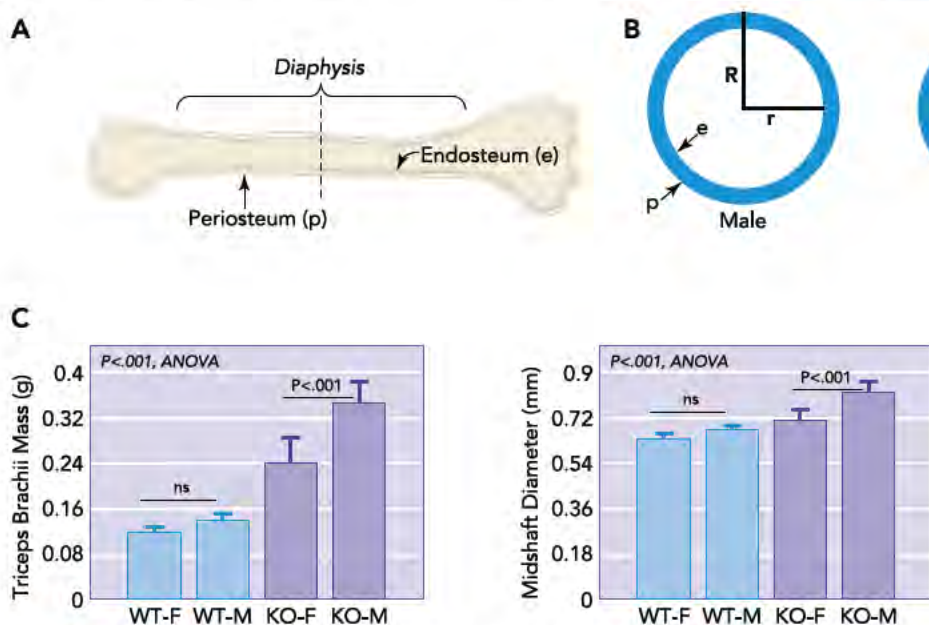

FIGURE 1. Schematic representation of a long bone and dimorphism in bone cross-sectional geometry

A: schematic representation of a long bone indicating the outer periosteal surface (p) and inner endocortical surface lining the medullary cavity (e), with a dotted line showing cross section. B: schematic representations of long bone cross-sectional geometry in males vs. females at the level of the dotted line shown in A. Males typically have a wider bone cross section and outer radius (R), whereas females have a smaller outer radius but thicker bone cortex, leading to a shorter inner radius (r). As women age, bone is resorbed from the endocortical surface so that older women must increase R to compensate for an increasing r. Males have a larger R and so are at lower fracture risk even as r increases. C: increased muscle mass in males is associated with increased bone diameter, even in mice. Male mice (M) lacking myostatin show a greater increase in forelimb triceps brachii mass than female (F) knockout (KO) mice, and forelimb bone (radius) diameter is largest in M mice lacking myostatin (KO) compared with wild-type (WT) mice.

gained from exercise when young can confer improved mechanical properties of bone with aging (76). The bone cortex also has an inner endosteal lining of bone-forming cells that deposits bone, increasing cortical thickness and reducing the cross-sectional area of the medullary cavity (FIGURE 1B). Men typically deposit a greater portion of bone periosteally and thus have relatively wider bones, whereas women deposit more bone endocortically and therefore have bones that are more slender but with a thicker cortex (FIGURE 1B). Bone resorption with aging normally occurs along the endocortical surface so that older, postmenopausal women have bones with a thinning cortex and small outer diameter, predisposing them to risk of fracture (63). Periosteal apposition continues into old age; however, the rate of apposition would need to increase close to 350% to offset the decline in bone strength due to gradual loss of bone on the endosteal surface (39).
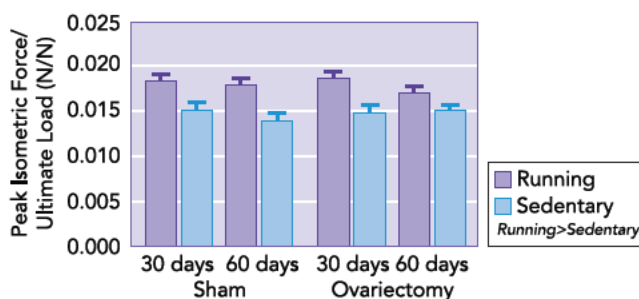
A Muscle Disease
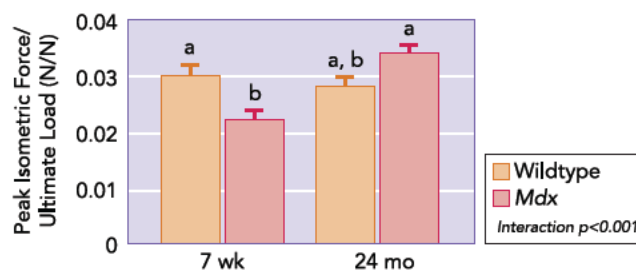
B Exercise

FIGURE 2. Ratio of muscle maximal isometric tetanic force to ultimate load as a function of muscle disease, physical activity, and estrogen
A: at the peak of muscle-disease onset (i.e., 7 wk of age), a mismatch is present between muscle and bone, such that the bone is stronger (i.e., determined by ultimate load during three-point bending) than the adjacent musculature (i.e., strength determined by in vitro assessment of isolated muscle contractility). With aging, the muscle-to-bone ratio remains constant in healthy wild-type mice. *Mdx* mice become undistinguishable from 24-mo-old wild-type mice, which is attributed to an improvement in muscle function with age. Data are means (SD) and are unitless. Statistically significant effects are indicated within the figure legend. Significant interactions between genotype and age detected by Holm-Sidak post hoc tests ($P < 0.05$) are indicated by the lowercase letters above the bars; values with the same letter are not significantly different. *B*: however, the loss of estrogen does not influence the muscle-to-bone ratio; running increases the ratio between muscle and bone, suggesting a transient mismatch between muscle and bone. Values are means (SD) and are unitless. Figure was adapted with permission from Refs. 52, 78.

Muscle plays a critical role in the regulation of bone size. An excellent example of this association comes from the human upper limb, which is non-weight bearing and in the case of the non-dominant arm does not normally experience high mechanical loads. In older adults (~70 yr of age), the periosteal circumference of the radius from the non-dominant upper limb is strongly associated with forearm muscle cross-sectional area, such that greater muscle size is consistently associated with greater periosteal circumference (16). Notably, the association between bone size and muscle cross-sectional area is stronger than the association between bone size and body weight. A similar association is observed in adolescents (57), where cross-sectional area and strength of the non-dominant radius is more strongly associated with forearm muscle cross-sectional area than with fat mass. An interesting comparative example in this regard can be observed in hypermuscular mice lacking myostatin, where larger forelimb muscles in male and female knockout mice are associated with increased midshaft diameter of the radius (FIGURE 1C). In these mice, midshaft diameter of the radius is more highly correlated with triceps brachii mass ($r^2 = 0.64$) than with body weight ($r^2 = 0.31$).

Impact of Aging on the Muscle-Bone Relationship

If the relationship between muscle and bone is indeed mechanical, then the ratio of muscle to bone properties (i.e., functional capacity or size) should remain relatively constant across adulthood. That is, anabolic or catabolic changes within muscle and bone would be expected to occur in parallel. Alternative patterns of change to the muscle-to-bone ratio (i.e., an increase or decrease) with aging could suggest that the functional capacity and/or size of muscle and bone are capable of being mismatched. There have been a few trials in mice to test the mutability of Wolff's Law and the mechanostat model by attempting to disrupt the scaling of mechanical properties in bone to those in muscle. Specifically, our group has investigated how the ratio of muscle function (i.e., peak isometric force assessed in vitro) to bone strength (i.e., ultimate load assessed by three-point bending) differs between young and aged mice with and without a genetic condition mimicking Duchenne muscular dystrophy (i.e., *mdx* mice) (FIGURE 2; Ref. 52). The muscle-to-bone ratio was not different between young and aged wild-type mice (FIGURE 2A). The ratio was, however, greater in aged compared with young *mdx* mice, with this being attributed to the active disease state in the young mice and muscle strength subsequently being impaired (52). In the aged *mdx* mice, muscle

strength had normalized, and the muscle-to-bone ratio was increased such that it was not different from that of wild-type mice (52).

We and others have also assessed the effects of physical activity/training (78) and estrogen deficiency (77, 78) on the muscle-bone relationship. In the study by Warren et. al. (78), this was done by assigning female mice to voluntary wheel running or sedentary groups, either with or without ovariectomy surgery. Mice that took part in wheel running as a form of exercise, had higher muscle-to-bone ratios compared with sedentary mice (FIGURE 2B) (78). This suggests that physical activity has the potential to disrupt the muscle-to-bone ratio, which would not be predicted by Wolff's Law. The removal of estrogen by ovariectomy for 30–60 days, however, did not disrupt the muscle-bone relationship (77, 78). Overall, these animal studies suggest that physical activity and muscle disease can create transient mismatches in the muscle-to-bone ratio, and perhaps that the influence of estrogen in mediating this response may be minimal.

To better assess the impact of aging on the muscle-bone relationship in humans, we conducted a systematic revew (Table 1). The bone outcome measure desired for a study's inclusion was bone mechanical strength, but because this is never reported, surrogates for bone strength were permitted. These surrogates included: estimates of bone strength (i.e., strength strain index), bone area (i.e., cortical bone area or cross-sectional area as determined by MRI or computed tomography), and bone mass [i.e., bone mineral content (BMC) or bone mineral density (BMD)] determined by DXA. The preferred muscle outcome measure was contractile strength, but measures of muscle anatomical dimensions (i.e., cross-sectional area or width) or mass were also permitted as a substitute. Studies were excluded if 1) the studied muscles or muscle groups were not in close proximity to bone that was studied or 2) insufficient data were available to calculate the ratio of muscle to bone outcome measures for each age group (i.e., only the correlation between the bone and muscle measures was reported, subjects were grouped based on age plus factors other than age). Fifteen studies met the inclusion and exclusion criteria. Mean muscle-to-bone ratios were calculated for each age group by dividing the reported mean muscle outcome measure by the corresponding mean bone outcome measure; standard deviations for these ratios were calculated using propagation of errors from the muscle and bone outcome standard deviations. Analysis of the ratio data was possible for all studies with the exception of Meema et al. (i.e., sample sizes lacking) (50), and thus for this study, the

aging pattern for the muscle-to-bone ratios is only described qualitatively.

Table 1 summarizes the 39 muscle-to-bone ratios from the 15 studies included in the systematic review. The ratio remained unchanged with aging in 19 of the 39 ratios, whereas 15 ratios decreased with age and 5 ratios increased (Table 1). To illustrate the variability among studies in the age-related ratio changes, three studies are discussed here in greater detail. These studies are those with the largest sample sizes, age ranges, and number of age groups compared (3, 50, 51). Atlantis et al. (3) was the largest study included in the analysis, and quantified muscle masses and BMC by DXA in over 1,000 men between the ages of 35 and 81 yr. The ratio of muscle mass to BMC decreased with aging in the arms but remained unchanged in the legs (Table 1). Melton et al. (51) provided raw data for 307 men and 345 women between the ages of 22 and 97 yr, which included lean mass of the arms and legs and a measure of femur bending strength index (i.e., flexural rigidity or EI) for each of the 652 participants (51). The muscle-to-bone ratio significantly decreased in men with aging but not in women ($P = 0.14$; Table 1). Meema et al. (50) reported bone mass of the radius and the width of the adjacent forearm musculature of the radius for 613 men and women across 11 different groups between the ages of 18–97 yr (FIGURE 3). Because the authors failed to report sample size for each of the 11 groups, this prevented running statistical analyses. However, it appears that the ratio increases with age in both men and women. Overall, these three studies highlight a high variability in how aging affects the muscle-to-bone ratio.

To determine whether a study's research design (i.e., the measures used to assess bone and muscle, number of age comparison groups, and age range among groups) affected how the muscle-to-bone ratio changes with aging, a logistic regression and a proportional odds (cumulative logit) model was utilized. The odds of an increase in the muscle-to-bone ratio with aging was dependent on the measures used to assess bone and muscle. For example, an increase in the ratio was >10-fold more likely to occur in studies in which bone was assessed via strength or BMC compared with when it was assessed via cross-sectional area (CSA). Similarly, an increase in the ratio was >50-fold more likely to occur in studies assessing bone via BMD compared with assessing bone via CSA. In contrast, studies that involved "strength" for the muscle strength measurement were more more likely to result in a decrease in the muscle-to-bone strength ratio with aging than the studies that used "area" for the muscle strength measurement. The number of age-comparison groups used in a study was related to the probability of observing an increase in the ratio. For each

Table 1. *Effect of aging on the muscle-to-bone ratio*

Study (Ref.)	Subjects/Age Range (yr)/Sample Size	Muscle Measure	Bone Measure	Effect of Aging on the Muscle-to-Bone Ratio
Heikkinen et. al., 1984 (30)	Men/31-75/142	KE strength (isometric)	Calcaneous BMD	Decreases
Hyakutake et al., 1994 (34)	Men/20-89/109	KE strength (90 deg/s)	Femur BMD	Decreases
		KF strength (90 deg/s)	Femur BMD	Decreases
	Women/20-79/231	KE strength (90 deg/s)	Femur BMD	Decreases
		KF strength (90 deg/s)	Femur BMD	Decreases
Calmels et. al., 1995 (9)	Women/44-87/101	KE strength (30 deg/s)	Femur BMD	Decreases
		KE strength (180 deg/s)	Femur BMD	No change
		KF strength (30 deg/s)	Femur BMD	Decrease
		KF strength (180 deg/s)	Femur BMD	No change
Humphries et. al., 1999 (33)	Peri- and post-menopausal women/45-65/96	KE strength (isometric)	Lumbar spine BMD	No change
Ryan et. al., 2004 (66)	Men/25-70/25	KE strength	Femur BMD	Increase
		Leg press strength	Femur BMD	No change
	Women/26-68/19	KE strength	Femur BMD	Trend for decrease
		Leg press strength	Femur BMD	No change
Sherk et. al., 2009 (70)	Men/18-64/68	DF/PF CSA (pQCT)	Tibia SSI	No change
		DF/PF CSA (pQCT)	Tibia cortical BMC	No change
		DF/PF CSA (pQCT)	Tibia cortical area	No change
		DF/PF CSA (pQCT)	Tibia cortical density	No change
Rice et. al. 1989, (61)	Men/25-90/20	EF CSA (CT)	Humerus CSA	Decreases
		EE CSA (CT)	Humerus CSA	Decreases
		PF CSA (CT)	Tibia CSA	Decreases
Overend et. al., 1992 (53)	Men/19-77/24	KE CSA (CT)	Femur CSA	Decreases
		KF CSA (CT)	Femur CSA	No change
Klein et. al., 2002 (43)	Men/20-90/33-44	EF/EE CSA (MRI)	Humerus cortical area	Decreases
		FF/FE CSA (MRI)	Radius Cortical Area	No Change
		FF/FE CSA (MRI)	Ulna Cortical Area	Trend for Decrease
McNeil et. al., 2009 (48)	Men/23-91/39	Total Leg Muscle CSA (CT)	Tibia CSA	Decreases
Horber et.al., 1997 (32)	Men/20-81/60	Arm lean muscle mass	Arm BMC	No Change
		Leg lean muscle mass (DXA)	Leg BMC	No Change
	Pre and post-menopausal women/20-79/59	Arm lean muscle mass (DXA)	Arm BMC	Trend for Increase
		Leg lean muscle mass (DXA)	Leg BMC	No change
Melton et. al., 2006 (51)	Men/20-93/307	Arm/leg lean muscle mass (DXA)	Femur EI strength	Decreases
	Women/21-97/345	Arm/leg lean muscle mass (DXA)	Femur EI strength	No change
Atlantis et. al., 2008 (3)	Men/35-81/1,068	Arms muscle mass (DXA)	Arms BMC	Decreases
		Legs muscle mass (DXA)	Leg BMC	No change
Sanada et. al., 2009 (67)	Women/20-76/138	Arm lean muscle mass	Arm BMC	Increases
		Leg lean muscle mass	Leg BMC	Increases
Meema et. al., 1973 (50)	Men/18-97/305	Muscle width of adjacent musculature	Radius bone mass	Increases*
	Women/18-90/308	Muscle width of adjacent musculature	Radius bone mass	Increases*

The review involved three steps. In *step 1*, using the search terms "muscle, bone, and aging" with delimitations to human studies and the English language, 1,097 articles were identified. Article titles and abstracts were reviewed in *step 2* to confirm that measures of bone and muscle functional capacity and/or structure were outcome measures in the studies, and that an assessment of aging was apparent; 119 studies met these criteria. In *step 3*, the following inclusion criteria for aging, bone, and muscle were applied during a full-text examination of the studies. A study's assessment of aging was deemed acceptable if a comparison was made across two or more age groups with an average age difference of at least 20 yr. KE, knee extensor muscles; BMD, bone mineral density; KF, knee flexor muscles; DF, dorsi flexor muscles; PF, plantar flexor muscles; CSA, cross-sectional area; SSI, strength strain index; EF, e bow flexor muscles; EE, elbow extensor muscles; FF, forearm flexors; FE, forearm extensors; BMC, bone mineral content; EI strength, flexural rigidity. *Statistical analyses were not performed on muscle-to-bone ratio due to missing sample sizes.

additional age group, the odds of observing an increase in the muscle-to-bone ratio with aging was reduced by half. These results indicate that study research design features do influence how the muscle-to-bone ratio changes with aging. However, for all of the research design features we considered, we found that they could account for no more than 30% of the variance in whether a muscle-to-bone ratio increased, decreased, or did not change with age. The large unaccounted for proportion of variance

highlights the potential for existence of one or more non-mechanical muscle-bone relationships.

Cellular and Molecular Mechanisms Underlying Age-Related Changes in the Muscle-Bone Relationship

The data reviewed above indicate that mismatches can occur between muscle and bone emanating from age-associated structural and/or functional changes in one tissue vs. another. Such dissociations are also observed with exercise and aging, where it is well known that exercise can significantly enhance bone mass and strength in young animals but not in older, adult animals, even when controlling for the duration and intensity of exercise (18, 55). There are likely a number of mechanisms that underlie this dissociation (FIGURE 4). First, there are changes in bone with aging that likely attenuate its response to muscle-derived stimuli. Osteocytes trapped within the mineralized matrix of bone are the key mechanostransducers in bone tissue, and osteocyte number and density decline with age, resulting in an increased number of empty lacunae in bone (8). These age-associated changes in the lacunocanalicular system of bone ultimately impair the bone signaling network (8, 74) and may deleteriously impact the diffusion of important growth factors and signaling molecules to target cells (FIGURE 4). On the other hand, a greater density of empty osteocyte lacunae has also been observed closest to the periosteal surface (29), and so periosteal expansion with increasing age could be related to an absence of osteocytes secreting the anti-osteogenic factor sclerostin. There are also changes in the periosteum that occur with aging that may limit the potential of muscle to increase bone size. Periosteum has been observed to become thinner and less cellular with age in rodents and other mammals (17), aged rodent periosteal cells show an impaired regenerative capacity and impaired response to parathyroid hormone (83), periosteal osteoblasts demonstrate an impaired capacity to proliferate in response to mechanical loading (49), and human periosteal cells demonstrate a lack of spontaneous cartilage formation in vitro after age 30 (14). Aged osteoprogenitor cells also show an attenuated response to growth factors (FIGURE 4). For example, the dose of IGF-1 required to elicit a mitogenic response in aged osteoprogenitor cells is much greater than that required for younger cells (71).

There are well documented changes in muscle that occur with aging, including an overall decrease in myofiber size, a decline in the number of excitable motor units within muscle, and the gradual infiltration of muscle with fatty tissue (73). There are also pronounced degradative changes in the neuromus-

cular junction with aging, which is known to contribute to synaptic loss (10) and may be related to oxidative stress and mitochondrial dysfunction (37). All of these changes will alter the contractile behavior of aged muscle, which as noted above could be an important mechanistic link between muscle and bone formation (FIGURE 4). One of the most exciting new developments in the area of muscle-bone interactions is the recognition that skeletal muscle secretes a variety of peptides collectively termed myokines (56), and a number of these myokines are well recognized as having positive effects on bone formation (26–28), as well as inhibitory effects on osteoblast differentiation (40). Importantly, muscle contraction stimulates the release of myokines both in vivo and in vitro (60, 68). IGF-1 expression in skeletal muscle tissue is elevated with muscle contraction (1), and circulating levels of IGF-1 are also increased with resistance exercise (63). Recent experiments also have shown that conditioned medium
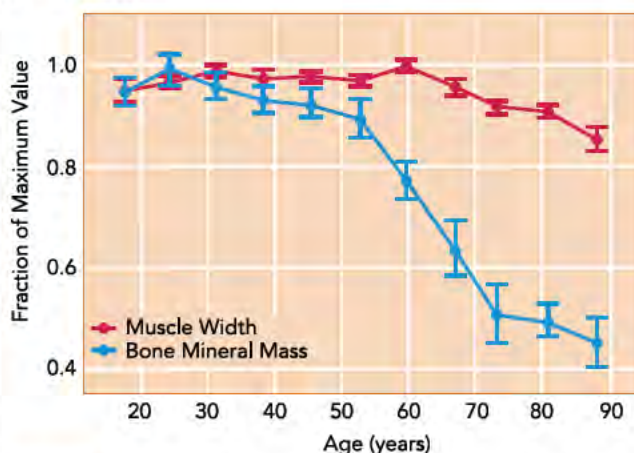
A Men
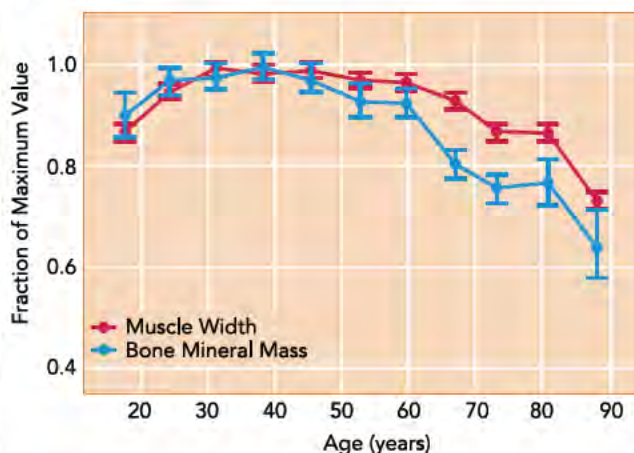
A Women

FIGURE 3. Aging-related changes in bone mineral mass of the radius and muscle width in the forearm in adult men and women
The figure was adapted with permission from Ref. 50, and data were normalized to the peak value for bone and muscle across the lifespan to show relative changes across the lifespan for men (A) and women (B).

from whole muscle explants stimulated in vitro can prevent the death of osteocytes when exposed to glucocorticoids (36), suggesting again that muscle contraction leads to the release of myokines that are beneficial for bone. Muscle contraction may also suppress the expression of factors that can inhibit the bone formation. For example, myostatin (GDF-8) expression is downregulated following concentric and eccentric muscle contractions (31), and myostatin suppresses the proliferation of bone marrow-derived stem cells in vitro (6).

Aging has been shown to reduce the anabolic effects of resistance exercise on muscle protein synthesis (23) and thus could potentially affect myokine synthesis and secretion. The effect of myokines on bone may be systemic as well as local, since elevated secretion of muscle-derived IL-15 not only decreases body fat but also increases whole-body bone mineral content (59). Another potential alteration in muscle with aging is that the myokine secretory profile may be altered. Aged human myoblasts, for example, show increased levels of TGF-β1 secretion in vitro compared with younger myoblasts (2), although it is not known how aging alters the secretion of other myokines with muscle contraction. In addition, aging is generally associated with a preferential denervation of fast-twitch fibers along with reinnervation of some of those fibers by α-motoneurons from slow motor units (58). It is not known how this could effect myokine secretion, but single fiber maximal isometric force production, either absolute or normalized to cross-sectional area, is not consistently different be-

tween slow- and fast-twitch fibers in older humans (e.g., Refs. 20, 72). It is well recognized that the bone marrow cavity accumulates fat with age (64), and it is certainly possible that increased bone marrow adipogenesis may attenuate the effects circulating myokines on endocortical (endosteal) osteoprogenitor cells (FIGURE 4). It should be noted that many of the associations between myokines and bone formation are more correlative and hypothetical rather than mechanistic. For example, although TGF-β1 and IL-6 are both well established myokines, their size may prevent their diffusion through the periosteum (44). Studies need to be performed to determine how muscle-specific alteration of myokine expression and/or targeted changes in myokine receptors in osteoprogenitor cells impact bone modeling and remodeling.

Summary and Conclusions

Aging is associated with the development of sarcopenia in skeletal muscle and osteoporosis in bone; however, it is not fully understood how the relationship between the two tissues is impacted by aging. Our experiments in mice and our review of human studies demonstrate that the relationship between muscle and bone size and strength changes significantly with age, so that a potential mismatch occurs between these tissues. There are pronounced cellular and molecular changes in bone and muscle cells with age that likely underlie these observations. Such changes include the loss of osteocytes in bone matrix
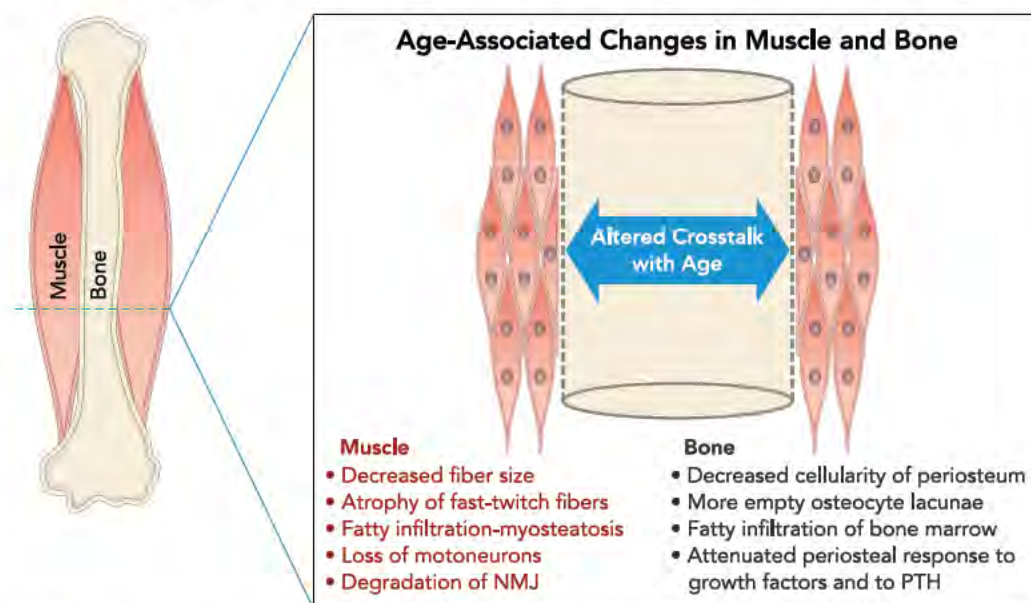
Age-Associated Changes in Muscle and Bone

Altered Crosstalk with Age

Muscle
- Decreased fiber size
- Atrophy of fast-twitch fibers
- Fatty infiltration-myosteatosis
- Loss of motoneurons
- Degradation of NMJ

Bone
- Decreased cellularity of periosteum
- More empty osteocyte lacunae
- Fatty infiltration of bone marrow
- Attenuated periosteal response to growth factors and to PTH

FIGURE 4. Schematic cross-section at the midshaft of a long bone diaphysis showing a summary of age-associated changes in muscle and bone
Muscle changes (red) include decreased fiber size, atrophy of fast-twitch fibers, fatty infiltration of muscle tissue, loss of motoneurons, and degradation of the neuromuscular junction (NMJ), all of which can negatively impact force production and potentially the secretion of myokines. Changes in bone (black) include decreased cellularity in the periosteum, loss of osteocytes in bone matrix due to senescence, fatty infiltration of bone marrow, and attenuated periosteal response to growth factors and parathyroid hormone (PTH). All of these changes can impair the capacity of bone to respond to anabolic stimuli.

and a decline in the proliferative capacity of osteoprogenitors in the periosteum, which attenuate the response of bone to muscle contraction and normal mechanical stimuli. Loss of motoneurons, reduced fiber size due to decreased muscle protein synthesis and increased degradation from atrogens, and perhaps fatty infiltration in muscle with age all negatively impact the contractile machinery and force-generating capacity of aged muscle. These data indicate that a therapeutic approach for improving bone health will require targeting both muscle and bone; that is, the changes in bone discussed above suggest that improvements in muscle function with age are unlikely to elicit an adequate anabolic stimulus in bone. Rather, strategies to enhance the anabolic capacity of periosteum in conjunction with therapeutics to increase the force- and power-generating capacity of aged muscle could significantly improve musculoskeletal health and function in the elderly. ∎

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REVIEW

The skeletal muscle secretome: an emerging player in muscle–bone crosstalk

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In vitro and *in vivo* studies provide evidence that a variety of growth factors and cytokines are actively secreted by muscle tissue. Muscle can therefore function as an endocrine and paracrine organ. These peptides characterize the muscle secretome, and many muscle-derived factors such as insulin-like growth factor-1, basic fibroblast growth factor, interleukin-15, myostatin and secreted protein acidic and rich in cysteine (osteonectin) are also known to have significant effects on bone metabolism. The factors secreted by muscle may vary according to muscle activity, in that muscle contraction, muscle atrophy or traumatic muscle injury can alter the type and relative abundance of particular factors released from muscle cells. The molecular and cellular pathways by which muscle-derived factors affect different types of bone cells (for example, osteoblasts, osteoclasts and osteocytes) are, however, poorly understood. Nevertheless, these findings further underscore the complex nature of muscle–bone interactions, and highlight the importance of integrating muscle biology and physiology into our understanding of bone growth, development and aging.

Introduction

Evidence from numerous studies has revealed for decades, if not centuries, that a close functional and developmental relationship exists between muscle and bone mass. Embryonic muscle paralysis and abnormal myogenesis lead to bones that are poorly mineralized and lack normal curvature,[1] and muscular dystrophies are associated with relatively low bone density and an increased incidence of bone fractures.[2] Loss of muscle mass with age, and the gradual infiltration of muscle with adipose tissue (myosteatosis), have been implicated in age-related bone loss and an increased risk of falls and fractures.[3] Moreover, muscle paralysis using agents such as botulinum toxin induces bone loss[4] and impairs fracture healing.[5] On the other hand, significant increases in muscle mass with myostatin deficiency are associated with larger muscle attachment sites and increases in bone cross-sectional area.[6,7] Historically, a number of different biomechanical and physiological mechanisms have been presented to explain the underlying relationship(s) between muscle function and bone metabolism. These range from mechanical models linking loading rates and strain history with bone adaptation,[8,9] to others linking muscle contraction with changes in fluid flow within bone tissue, which can in turn regulate bone formation.[10]

The majority of models, including those cited above, that link changes in muscle mass with alterations in bone formation and strength appropriately emphasize the important role of muscle contraction in generating mechanical stimuli for bone. Hence, the muscle–bone relationship is in large part a mechanical one, with muscle being the key driver in this relationship via the contractile forces that it imposes upon bone tissue. Indeed, it is clear from numerous studies that mechanical stimulation is important for bone health, and that exercise-induced muscle contraction may enhance bone mass during growth. There is, however, evidence that muscle tissue itself may have positive effects on bone formation and bone repair independent of mechanical stimulation. For example, it is well established in the clinical literature that covering bone fractures with muscle flaps improves fracture healing in cases of traumatic orthopaedic injury,[11–13] and that implants of muscle alongside periosteum can stimulate new bone formation directly.[14] Similarly, muscle damage and trauma to muscle surrounding bone defects, can impair and delay bone healing.[15,16] These findings provide additional support for the concept that health and viability of the muscle bed are key for normal bone formation and bone repair.[17–19]

The fact that muscle flaps alone can enhance bone formation suggests that muscle may serve as an important collateral source of blood for adjacent bone tissue,[16] and perhaps as a source of trophic factors.[14,17] This review focuses on the

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latter hypothesis for the following reasons. First, it is clear that intact muscle flaps are a rich source of secreted growth factors.[20] Second, although muscle does provide important vascular support for bone, skin has a higher vascular density than muscle, yet fasciocutaneous skin flaps do not have the same anabolic effects on healing bone as muscle flaps.[21] Third, it is clear that conditioned medium from cultured muscle cells has positive effects (for example, increasing extracellular matrix synthesis) on cultured chondrocytes,[22] revealing that muscle is a source of secreted growth factors both *in vivo* and *in vitro*. Finally, the muscle secretome has become increasingly well characterized.[23–27] We now know that muscle secretes a wide variety of growth factors, cytokines and molecules involved in extracellular matrix remodeling. Moreover, different laboratories have independently identified the same factors secreted from muscle, further validating the existence of a well-defined muscle secretome. Some of these factors have been referred to as 'myokines' by different authors;[28–30] however, several muscle-derived factors (for example, IGF-1, FGF-2, IL-6) are secreted in abundance by other tissues, so the term myokine should not be taken to imply that they are muscle-specific.

Our growing knowledge of the muscle secretome has important implications for bone biology, as it presents new opportunities for targeting muscle in order to better develop the therapeutic program for aging and healing bone. This review seeks to highlight the muscle-derived factors that may impact bone metabolism, and also propose future directions for research aimed at advancing our current understanding of muscle–bone crosstalk.

Growth Factors Actively Secreted by Skeletal Muscle

Insulin-like growth factor-1 (IGF-1). Wound exudates from intact muscle flaps contain high levels of IGF-1,[20] and immunohistochemistry has been used to localize IGF-1 along the muscle-periosteal interface of mouse forelimbs.[25] These *in vivo* findings are consistent with *in vitro* studies in which proteomic approaches have been used to detect IGF-1 in conditioned medium from cultured myotubes.[23,27,31] IGF-1 expression, measured as an increase in IGF-1 mRNA, is increased in skeletal muscle with muscle contraction,[32] and elevated levels of IGF-1 protein are detected in serum following resistance exercise.[33] It is well established that IGF-1 has important osteogenic effects on the skeleton and IGF-1 is also involved in myofiber hypertrophy, suggesting that muscle-derived IGF-1 may couple both muscle and bone anabolism (**Figure 1**).[30]

Basic fibroblast growth factor (FGF-2). FGF-2 lacks the conventional signal sequence for export out of cells via the classic exocytotic pathway, and it has been shown that mechanically induced plasma membrane disruption is one mechanism by which FGF-2 is released from myocytes both *in vivo* and *in vitro*.[34,35] Eccentric, lengthening contractions are particularly effective for releasing FGF-2 stored in the cytosol of muscle cells,[35] and levels of FGF-2 detected in conditioned medium from cultured myotubes are increased with mechanical stretching.[34] It is important to note here that the muscle 'injury' that occurs during eccentric muscle contraction involves plasma membrane disruptions that are followed by membrane repair.[36] These types of mechanical disruptions are argued here to involve a different cascade of molecular signaling events than
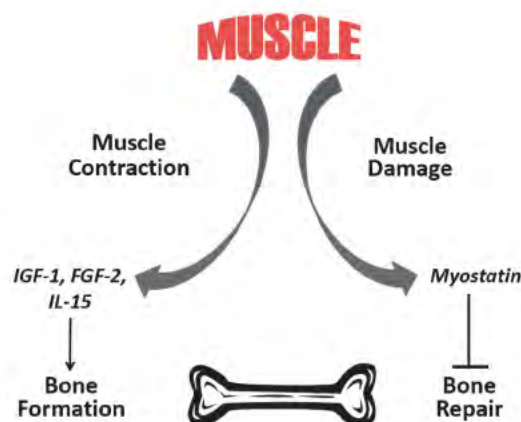

Figure 1 Resistance exercise and eccentric muscle contraction induce the secretion and release of the osteogenic factors IGF-1 and FGF-2 from skeletal muscle. In contrast, traumatic muscle injury and perhaps systemic inflammation and disuse increase the secretion of myostatin from skeletal muscle, which in turns impairs chondrogenesis and bone healing.

traumatic muscle injuries that are associated with muscle regeneration. The latter type of mechanical and structural disruption is much more severe, and involves cleanup of necrotic tissue by macrophages, expression of inflammatory factors and activation of satellite cells. FGF-2 has positive effects on bone formation in estrogen-deficient rodents and is a well-known osteogenic factor.[25,30] Mechanically induced release of FGF-2 following eccentric contraction and plasma membrane disruption is another potential pathway by which physical activity and bone formation may be coupled physiologically (**Figure 1**).[25,30]

Myostatin (GDF-8). Although factors such as IGF-1 and FGF-2 are secreted by a number of tissues in addition to muscle, myostatin is most abundant in muscle tissue and appears to be secreted primarily by muscle. Thus, myostatin can be considered a bona fide myokine. Conditions associated with elevated levels of myostatin expression include disuse atrophy, cancer- and AIDS-related cachexia and increased circulating levels of glucorticoids.[30] Myostatin treatment induces muscle wasting and myostatin deficiency increases muscle mass. We have recently shown that myostatin is highly expressed by injured myofibers following traumatic extremity injury,[37] and that local application of exogenous myostatin increases skeletal muscle fibrosis and inhibits bone repair.[37] These data are consistent with studies referenced above, demonstrating that intact muscle flaps enhance bone repair, whereas coverage of bone injuries with damaged muscle does not have the same positive effects on bone healing. These *in vivo* data are also consistent with our *in vitro* studies showing that myostatin treatment suppresses the proliferation and chondrogenic differentiation of bone marrow-derived stromal (stem) cells.[38] In addition, blocking the myostatin using a recombinant propeptide improves muscle regeneration and fracture healing following orthopaedic trauma.[39] Although severe muscle trauma and muscle damage increase myostatin expression, which in turn impairs bone healing, eccentric muscle contraction and exercise both decrease myostatin expression in skeletal muscle.[40–42] These studies suggest that although IGF-1 and FGF-2 are muscle-derived factors that can have significant, positive effects on bone formation, myostatin is a factor released from muscle during traumatic

Table 1 Growth factors, cytokines and other peptides secreted by muscle, the factors that influence their secretion and their potential effects on bone metabolism

Muscle-derived peptides	Factors that stimulate peptide secretion	Role(s) in bone metabolism
Growth factors		
IGF-1	Resistance exercise	Stimulates bone formation
FGF-2	Eccentric muscle contraction	Stimulates bone formation
GDF-8	Muscle damage, cachexia, atrophy	Suppresses chondrogenesis and fracture healing
Extracellular matrix molecules		
SPARC	Resistance exercise, muscle regeneration	Promotes bone mineralization
MMP-2	Resistance exercise and re-loading	Fracture callus remodeling, bone formation
BMP-1	Blast trauma to muscle	Cleaving of procollagen and possibly heterotopic ossification
Inflammatory cytokines		
IL-6	Physical activity and muscle contraction	Bone resorption and turnover
IL-7	Physical activity and muscle contraction	Bone resorption
IL-15	Resistance exercise	Increase bone mass, decrease adiposity

Abbreviation: IL, interleukin.

and catabolic conditions that may inhibit and suppress bone repair (**Figure 1**).[30]

Factors Involved in Extracellular Matrix Remodeling Secreted by Muscle

Secreted protein acidic and rich in cysteine (SPARC, or osteonectin). SPARC is a glycoprotein that is abundant in the extracellular matrix of various tissues including bone and skeletal muscle and is involved in tissue repair, remodeling of the extracellular matrix, and promoting collagen mineralization by osteoblasts.[43] One of the more surprising aspects of research on the muscle secretome is the consistency with which SPARC is detected as a factor secreted by isolated muscle cells. A number of different research groups using both human and rodent-derived muscle cells have identified SPARC in conditioned medium from cultured myotubes.[23,24,26,31] SPARC secretion is increased following resistance exercise and myotube hypertrophy,[26] but SPARC is also highly expressed following injury and during muscle regeneration.[44] Additional research is needed to determine the role of muscle-derived SPARC in bone formation, but the studies reviewed above suggest that exercise-induced SPARC secretion could potentially have a role in enhancing bone formation and mineralization (**Table 1**).

Matrix metalloproteinase-2 (MMP-2). Mice lacking MMP-2 are known to show bone loss and reduced bone density, and absence of MMP-2 effects later stages of fracture callus remodeling.[45] MMP-2, similar to SPARC, is found in both muscle as well as bone, and studies using cultured myotubes reveal that MMP-2 is actively secreted by myotubes *in vitro*.[23,24] Insulin treatment of rat myotubes increases secretion of MMP-2, and serum levels of MMP-2 are elevated in diabetic patients suffer-

ing from hyperinsulinemia.[46] MMP-2 expression increases with exercise[47] and with re-loading following hindlimb suspension,[48] and decreases with injury,[49] but is elevated in skeletal muscle following disuse.[50] These data show that MMP-2, like SPARC, has an important role in muscle tissue remodeling during various repair events. The role of muscle-derived MMP-2 in bone metabolism is also unclear, but muscle-derived MMP-2 could potentially couple both muscle and bone turnover (**Table 1**).

Bone morphogenetic protein-1 (BMP-1). BMP-1 is not a true bone morphogenetic protein but rather is a protease that cleaves the propeptide fragments of procollagens I, II and III.[51] BMP-1 is secreted from cultured primary human myotubes *in vitro*,[31] and its secretion is decreased in rat myotubes exposed to very high contractions of insulin.[46] Recently, high levels of BMP-1 protein and mRNA were detected in muscle biopsies from patients who had experienced blast trauma in the combat setting.[52] This is significant from the perspective of muscle–bone crosstalk because blast trauma is associated with a high incidence of heterotopic ossification, a condition where bone forms within muscle tissue.[52] Additional work is needed to better understand the role(s) of muscle-specific BMP-1 secretion in normal and pathological bone formation, but BMP-1 could represent a potential therapeutic target for the prevention of heterotopic ossification (**Table 1**).

Inflammatory Factors that are Secreted from Muscle During Exercise

Interleukin-6, -7 and -15 (IL-6, IL-7, IL-15). The term 'myokine' was originally coined in reference to IL-6, a factor that Pedersen and colleagues[28,53,54] determined was released from muscle during exercise, and had important effects on other tissues including the liver and adipose depots. Type I (slow-twitch) fibers express high levels of IL-6, and IL-6 levels are increased in serum with exercise.[53] IL-6 can stimulate expression of the anti-inflammatory factor IL-10, and mice lacking IL-6 develop obesity and insulin resistance.[55] IL-6 is often considered a pro-resorptive cytokine for bone, but mice lacking IL-6 do not show an osteopenic phenotype and IL-6 may facilitate bone formation during conditions of high bone turnover.[56,57] IL-7 is also widely considered to be an osteoclastogenic cytokine,[58] and IL-7 is actively secreted by muscle cells.[59] Interestingly, many of the studies cited above (for example, Chan *et al.*[24], Norheim *et al.*[26], Henningsen *et al.*[27], Yoon *et al.*[46]) that utilized *in vitro* cultures of myotubes to characterize the muscle secretome failed to identify IL-6 and -7 in conditioned medium. These observations raise the possibility that there are other myokines left to be identified that may be discovered through novel alternative *in vivo* and *in vitro* approaches. Finally, IL-15 is highly expressed in muscle tissue and is upregulated following resistance exercise.[60] Transgenic mice overexpressing IL-15 in skeletal muscle that show elevated circulating IL-15 levels also show decreased fat mass and increased bone mass.[61] Importantly, these mice did not differ in lean mass or body weight from normal controls, suggesting that the increased bone mass was not due to any alterations in mechanical factors.

Summary and Future Research

A paracrine role for skeletal muscle is not necessarily a new concept, as experiments where skeletal muscle was trans-

planted into cardiac tissue have revealed that skeletal muscle implants are a source of trophic factors supporting the survival of surrounding myocardial cells.[62] The paracrine and endocrine effects of muscle on bone are, however, only now beginning to become more well defined. We are at a very early stage in our understanding of how the muscle secretome impacts bone and other organs, and hence a number of outstanding questions remain. These include questions as to how muscle-derived factors impact particular cell types. For example, how do factors secreted from myocytes during muscle contraction and muscle hypertrophy influence bone resorption by osteoclasts, bone formation by osteoblasts or the adipogenic differentiation of bone marrow-derived stem cells? Similarly, how do various modes of muscle contraction, such as concentric vs eccentric contraction, alter myokine expression or secretion? Finally, how do unloading, microgravity or prolonged bedrest impact the muscle secretome?

These questions all point to the larger issue of how muscle activity and metabolism impact the overall systemic environment to which cells of bone and other tissues are exposed. For example, it is clear that exposing muscle of aged animals to circulating factors from younger animals improves the regenerative capacity of muscle.[63] Alternatively, how do changes in muscle, be they catabolic or anabolic, alter the systemic milieu to affect other organs such as bone? Preserving muscle mass decreases mortality and improves survival in tumor-bearing mice,[64,65] and loss of lean mass is an important predictor of health outcomes following burns and with chronic wounds.[66,67] The positive effects of muscle mass on health outcomes are related at least to the fact that muscle is the primary source of free amino acids in the body;[68,69] however, it is also likely that certain myokines may also have a role. For example, treatment of a tumor cell-line with mouse serum following exercise, or with conditioned medium from myotubes following electrical stimulation, reduces tumor cell proliferation and increases apoptosis.[70] These effects of muscle-derived serum and media on tumor cells were attributed to the fact that myotubes secrete the anti-tumor protein oncostatin M.[71] This finding has significant implications for cancer and bone (for example, osteosarcoma), and future studies might be directed at elucidating the interface between myokines, metastasis of cancer to bone and tumor growth in bone tissue.

Another outstanding question concerns the interactions of muscle-derived factors with organs other than bone. Skeletal muscle hypertrophy and hyperplasia increase serum IGF-1 levels in mice lacking myostatin. The increase in circulating IGF-1 levels is associated with elevated liver-derived IGF-1 in these mice with no change in muscle-specific IGF-1 expression.[71] Thus, heritable variation in muscle mass has the potential to dramatically alter growth factor production by other organs, which is likely to have broad systemic effects on tissues such as bone and cartilage. Exercise has recently been shown to increase secretion of follistatin by hepatocytes,[72] and follistatin is a potent inhibitor of both activin and myostatin, factors that suppress muscle hypertrophy and impair muscle regeneration. Follistatin also increases osteoblast mineralization *in vitro*,[73] raising the possibility that interactions among myokines and hepatokines may influence bone metabolism both directly, through their effects on bone cells and indirectly, by modulating growth factor and cytokine production in other organs. A similar relationship may exist between

muscle and fat, where myokines can induce lipolysis,[74] that would presumably affect circulating levels of adipokines such as leptin, which can in turn significantly alter bone formation and resorption.

In conclusion, *in vivo* and *in vitro* studies now demonstrate that muscle can function as an endocrine and paracrine organ. The factors secreted by muscle may vary according to muscle activity, such as concentric and eccentric contraction, disuse or damage in the form of traumatic injury. Factors actively secreted by muscle range from growth factors to inflammatory cytokines, and these peptides have the potential to alter bone metabolism in a variety of ways. Additional research is needed to better define the molecular and cellular pathways through which muscle-derived factors affect different types of bone cells, and anabolic and catabolic processes, in bone tissue. Nevertheless, the studies reviewed here further underscore the complex nature of muscle–bone interactions, and highlight the importance of integrating muscle biology and physiology into our understanding of bone growth, development and aging.

Conflict of Interest

The author declares no conflict of interest.

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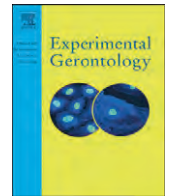
Effects of the activin A–myostatin–follistatin system on aging bone and muscle progenitor cells

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ABSTRACT

The activin A–myostatin–follistatin system is thought to play an important role in the regulation of muscle and bone mass throughout growth, development, and aging; however, the effects of these ligands on progenitor cell proliferation and differentiation in muscle and bone are not well understood. In addition, age-associated changes in the relative expression of these factors in musculoskeletal tissues have not been described. We therefore examined changes in protein levels of activin A, follistatin, and myostatin (GDF-8) in both muscle and bone with age in C57BL6 mice using ELISA. We then investigated the effects of activin A, myostatin and follistatin on the proliferation and differentiation of primary myoblasts and mouse bone marrow stromal cells (BMSCs) in vitro. Myostatin levels and the myostatin:follistatin ratio increased with age in the primarily slow twitch mouse soleus muscle, whereas the pattern was reversed with age in the fast twitch extensor digitorum longus muscle. Myostatin levels and the myostatin:follistatin ratio increased significantly ($+75\%$) in mouse bone marrow with age, as did activin A levels ($+17\%$). Follistatin increased the proliferation of primary myoblasts from both young and aged mice, whereas myostatin increased proliferation of younger myoblasts but decreased proliferation of older myoblasts. Myostatin reduced proliferation of both young and aged BMSCs in a dose dependent fashion, and activin A increased mineralization in both young and aged BMSCs. Together these data suggest that aging in mice is accompanied by changes in the expression of activin A and myostatin, as well as changes in the response of bone and muscle progenitor cells to these factors. Myostatin appears to play a particularly important role in the impaired proliferative capacity of muscle and bone progenitor cells from aged mice.

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1. Introduction

Aging is associated with a number of changes in the musculoskeletal system, including progressive deterioration of articular cartilage in the form of osteoarthritis, loss of muscle mass in the form of sarcopenia, and loss of bone density and strength in the form of osteoporosis. Muscle weakness and frailty contribute directly to postural instability, which in turn increases the risk for falls, and falls are the main etiological factor in more than 90% of bone fractures. The more than 1.5 million osteoporotic fractures a year in the US place significant burden on the healthcare system, and also contribute to significant morbidity and poor quality of life. Treatments that can improve muscle strength and at the same time increase bone mass will therefore significantly reduce fracture related morbidity and mortality.

The activin A–myostatin–follistatin system is believed to play an important role in musculoskeletal growth, development and aging.

Myostatin (GFD-8) and activin A bind type II activin receptors and signal through a transforming growth factor beta signaling pathway involving SMAD phosphorylation. Activin is thought to bind with greater affinity to the type IIA activin receptor (ActRIIA) and myostatin to the type II B receptor (ActRIIB), but both are involved in the regulation of muscle mass (Gilson et al., 2009; Lee et al., 2010). Follistatin antagonizes both myostatin and activin A activity, and mice overexpressing follistatin in skeletal muscle show a more dramatic phenotype than mice lacking myostatin alone (Lee et al., 2005). These data suggest that alterations in either myostatin or activin A with aging or disuse can have significant effects on muscle mass, and these may be further influenced by relative levels of follistatin. Although myostatin is not highly expressed by bone cells, loss of myostatin function is associated with increased bone density in mice (Elkasrawy and Hamrick, 2010; Morissette et al., 2009). The increased bone density of mice lacking myostatin is likely multifactorial, and may result not only from the indirect effects of increased muscle mass (Hamrick, 2011, 2012) but also from increased circulating levels of IGF-1 (Williams et al., 2011). The type IIA and type IIB activin receptors are both expressed by chondrocytes and osteoblasts, and activin A has been observed to inhibit mineralization by osteoblasts in vitro whereas follistatin can increase mineralization (Eijken et al., 2007). Furthermore, inhibiting activin A in

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vivo using a decoy soluble activin A receptor (ActRIIA) increases bone formation in mice (Pearsall et al., 2008).

The activin A myostatin follistatin system therefore appears to play a number of important roles in muscle as well as in bone metabolism. Although some studies have found no association between age and myostatin transcript levels in skeletal muscle (Marcell et al., 2001), others reveal a marked elevation in skeletal muscle myostatin expression with aging in humans (Leger et al., 2008). Additional research suggests that circulating levels of myostatin increase with age in men and women, and are highest in people aged 60 90 (Yarasheski et al., 2002). The latter finding may implicate myostatin in the sarcopenia of aging, hence myostatin inhibitors could be useful pharmacological agents for treating age related muscle atrophy as well as bone loss. Indeed, a myostatin inhibitor has been shown to improve muscle regeneration in aged mice (Siriett et al., 2007), and a recent study reveals that muscle derived stem cells from older male patients show a +65% higher level of myostatin expression compared to stem cells from younger patients (McKay et al., 2012). Myostatin levels can also be reduced in skeletal muscle with resistance exercise in older men (Dalbo et al., 2011), and aerobic exercise can decrease myostatin expression in older women (Konopka et al., 2010). While there has been considerable interest in the role of myostatin in musculoskeletal changes with aging, much less is known about activin A and follistatin. Circulating activin A levels have been observed to increase with age, whereas circulating follistatin levels did not show age associated changes (Baccarelli et al., 2001; Hurwitz and Santoro, 2004). Very little information exists, however, with regard to tissue specific changes in activin A and follistatin levels with aging or how the response of muscle and bone cells to these factors is altered with age.

We have previously shown that aged C57BL/6 mice share a number of key features in common with the aging human musculoskeletal system including an age related decline muscle mass, both absolutely and relative to body mass, as well as loss of bone density, bone formation, and bone strength (Hamrick et al., 2006). In order to address several of the questions outlined above we examined age related changes in the expression of activin A, follistatin, and myostatin in mouse skeletal muscle as well as in mouse bone marrow supernatant. We also investigated the response of primary muscle and bone cells from young (12 months) and aged (24 months) mice to activin A, follistatin, and myostatin treatment in vitro.

2. Materials and methods

2.1. Animals

C57BL6 mice were purchased from aged rodent colony at National Institute on Aging, National Institutes of Health (USA) at 12 and 24 months of age and delivered to Georgia Health Sciences University, Augusta, GA. Animals were allowed to acclimate for approximately two weeks and were maintained at the Laboratory Animal Service Facility of the Georgia Health Sciences University. Animals were sacrificed by

CO$_2$ overdose and thoracotomy following procedures approved by the Institutional Animal Care and Use Committee of Georgia Health Sciences University.

2.2. ELISA assays

Whole extensor digitorum longus or soleus muscle was dissected from C57BL6 mice at either 12 or 24 months of age and snap frozen in liquid nitrogen. Each muscle was placed in 1 ml phosphate buffered saline (PBS) and subjected to homogenization using Fisherbrand Tissuemiser® rotary homogenizer until large pieces of muscle were no longer visible. Samples were subjected to two freeze thaw cycles to disrupt the plasma membrane then centrifuged briefly. Samples were separated into 250 μl aliquots and stored at −80 °C until assayed. Follistatin and Activin A ELISA kits were purchased from R&D Systems and assays performed according to manufacturer's protocol without sample dilution. Myostatin ELISA kits were purchased from Alpco diagnostic and also performed according to manufacturer's protocol, but samples for myostatin assay were diluted 5 fold. Total protein was measured for each sample using Pierce BCA Protein Assay Kit (Thermo Scientific) according to manufacturer's protocol. Briefly: after thawing samples for ELISA, 25 μl of sample was incubated undiluted with 200 μl of Pierce® working reagent for 20 min at 37 °C. Absorbance was recorded at 590 nm wavelength and total protein concentrations were calculated based on standard curve generated with bovine serum albumin.

Left and right femora were dissected from C57BL6 mice at either 12 or 24 months of age and placed in PBS. Samples were immediately minced using fine point surgical scissors. Samples were vortexed vigorously and triturated with a pipette to liberate marrow from bone. Samples were centrifuged briefly, supernatant was collected, and bone fragments were discarded. Supernatant was centrifuged at
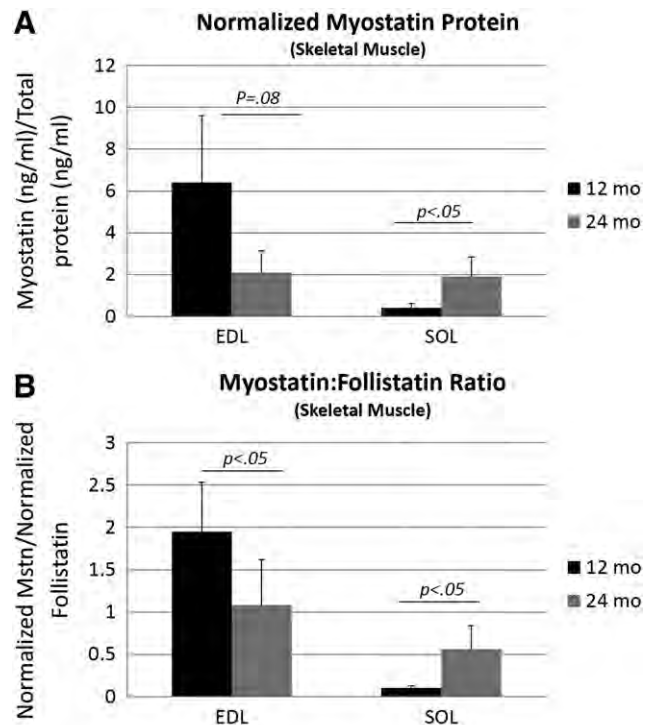

Fig. 1. Myostatin protein normalized to total protein (A) and the ratio of normalized myostatin to follistatin (B) in the extensor digitorum longus muscle (EDL) and soleus (SOL) of mice 12 months of age (12 mo) and 24 months of age (24 mo). Myostatin levels decline with age in the EDL but increase with age in the soleus. Error bars represent one standard deviation and sample size includes six replicates per group.

Table 1
List of oligonucleotide primer sequences for qRT-PCR.

Name	Sequence	Amplicon size
Myogenin	Fwd: GGAAGTCTGTGTCGGTGGAC	150
	Rev: CGCTGCGCAGGATCTCCAC	
MyoD	Fwd: GCCTGAGCAAAGTGAATGAG	184
	Rev: GGTCCAGGTGCGTAGAAGG	
Myosin, heavy polypeptide 3 (Myh3)	Fwd: ACAGTCAGAGGTGTGACTCAGCCG	90
	Rev: CCGACTTGCGGAGGAAAGGTGC	
BMP-2	Fwd: TGTTTGGCCTGAAGCAGAGA	83
	Rev: TGAGTGCCTGCGGTACAGAT	
Osteocalcin	Fwd: ATTTAGGACCTGTGCTGCCCTA	120
	Rev: GGAGCTGCTGTGACATCCATAC	

1500 g for 5 min to pellet cells. Supernatant was collected, aliquoted, and stored at −80 °C until assayed. Specific ELISA assays and total protein were performed and measured as described above for skeletal muscle homogenates.

2.3. Primary myoblast isolation, culture, and proliferation and differentiation assays

Mice 12 and 24 months of age were euthanized and tibialis anterior muscle dissected and place in sterile PBS. The muscle was minced with a sterile scalpel under aseptic conditions. Minced muscle was digested in 0.2% collagenase type II (Gibco) for 1 h with frequent shaking followed by digestion in 1× trypsin for 30 min. The slurry was pelletted and trypsin supernatant removed. The slurry was resuspended in proliferation medium. Upon completion of enzymatic digest, slurry was poured over a 70 μm cell strainer (Fisher) to remove any remaining connective tissue. The cells were then added to collagen type I (BD Bioscience) coated T 25 flasks.

Primary myoblasts were allowed to attach for 72 h. Cells were then maintained in proliferation medium (PM): DMEM (Hyclone) supplemented with 10% fetal bovine serum, 10% horse serum, 1%

penicillin/streptomycin, and 0.5% chick embryo extract (Sera Labs U.K.). Medium was changed every 48 h until T 25 flask was confluent. Once confluent, cells were trypsinized and counted using NucleoCounter (New Brunswick Scientific). Cells were then plated in a 96 well plate at 5000 cells/cm². Cells were allowed to attach in proliferation medium for 48 h. Proliferation medium was removed, cells washed with PBS, and DMEM supplemented with 1% insulin transferrin sodium selenite (ITS) was added followed by either control (PBS) or high or low dose activin A (50 ng/ml and 100 ng/ml), follistatin (100 ng/ml and 1000 ng/ml) or myostatin (100 ng/ml and 1000 ng/ml) (R&D Systems, Minneapolis). Doses follow those utilized by He et al. (2005) for activin A and Zhu et al. (2007) for myostatin and follistatin. After 24 h of treatment, MTS reagent was added according to the manufacturer's protocol (Promega, Madison) and absorbance at 492 nm was read 2 h later.

For differentiation assays, cells were isolated and cultured for one week until confluent as described above. Cells were then trypsinized and plated in 12 well plates at 5000 cells/ml and allowed to attach overnight in proliferation medium. PM was removed, cells washed with PBS, and DMEM supplemented with 1% ITS was added followed by the addition of Mstn, Fstn, Activin A or control. Cells were
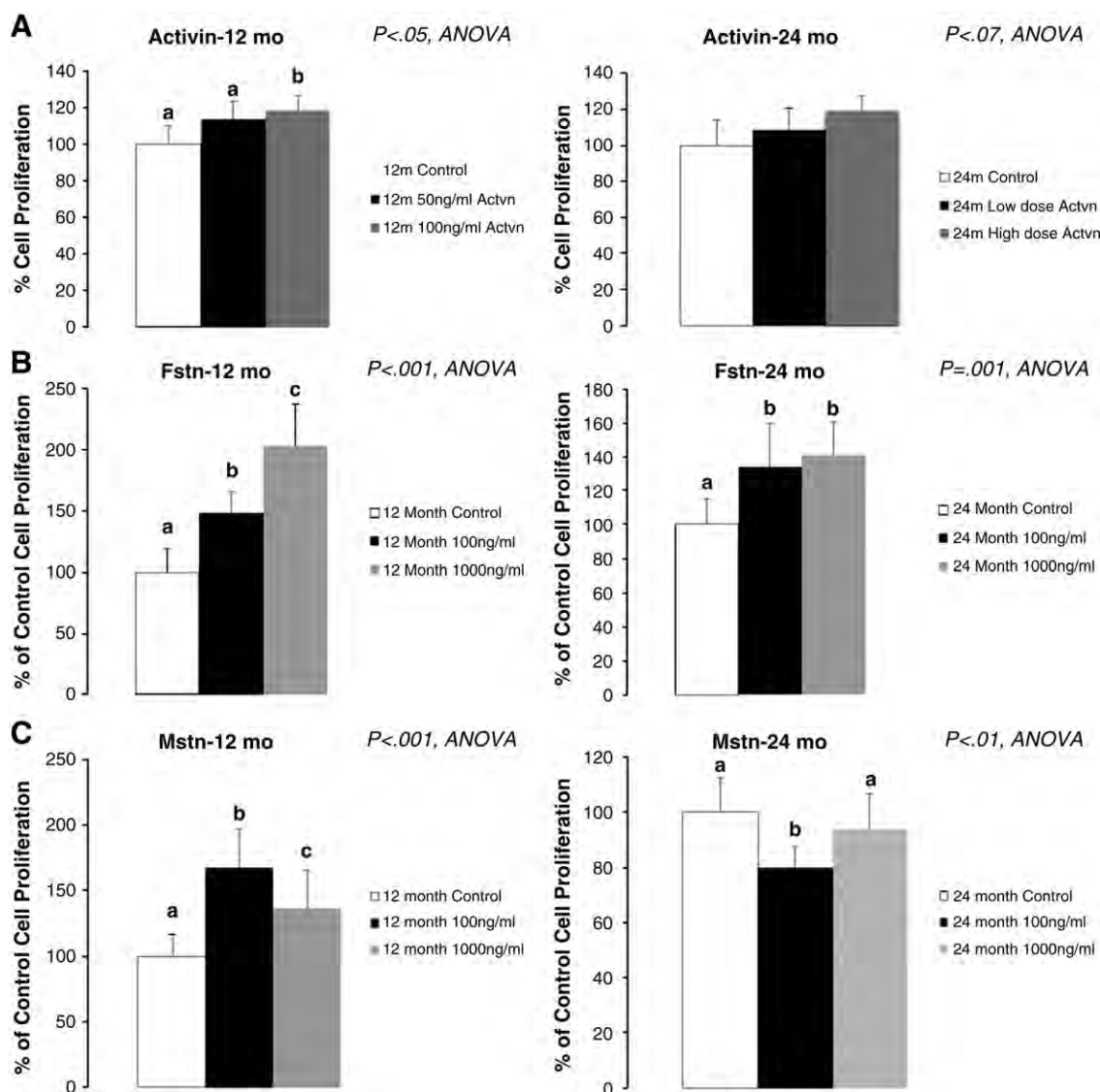

Fig. 2. Results of proliferation assays following treatment of primary myoblasts with activin A (A; activin), follistatin (B; Fstn), and myostatin (C; Mstn). Means with different superscripts differ significantly from one another (P<.05). Error bars represent one standard deviation and sample size includes eight replicates per group.

maintained in treatment for 48 h then harvested in TRIZOL® reagent (Invitrogen) for RNA isolation and subsequent cDNA synthesis (Bio Rad). 50 100 ng of cDNA was amplified in duplicates in each 40 cycle reaction using an iCycler™ (Bio Rad) with annealing temperature set at 60 °C, ABsolute™ QPCR SYBR® Green Fluorescein Mix (ABgene, Thermo Fisher Scientific), and custom designed qRT PCR primers (Table 1). A melt curve was used to assess the purity of amplification products. mRNA levels were normalized to β Actin/18S and gene expression was calculated as fold change using the comparative CT method. If not otherwise indicated, treated groups were compared to PBS control groups.

2.4. Primary bone marrow stromal cell (BMSC) isolation, culture, and proliferation and differentiation assays

Bone marrow aspirates were flushed from mouse femora and tibias and BMSCs isolated using magnetic bead sorting as previously described (Zhang et al., 2008). Briefly, magnetic nanoparticles conjugated to anti mouse CD11b, CD11c, CD45R/B220, and Pan DC monoclonal anti bodies were used to remove hematopoietic lineage cells and those that were negative for these four antigens remained in the solution and were collected and subjected to a round of positive selection using anti Sca 1 microbeads.

Enriched BMSCs were cultured in proliferation medium (DMEM supplemented with 10% heat inactivated FBS) in T 75 flasks until ~80% confluent. Cells were then lifted with trypsin/EDTA, plated in 96 well plates at a density of 5000 cells/well in proliferation medium,

and allowed to attach for 24 h. Proliferation medium was removed, cells washed with PBS, and DMEM supplemented with 2% heat inactivated FBS (for BMSCs) was added followed by control (PBS), activin A, follistatin, or myostatin (all from R&D Systems, Minneapolis) at the same doses noted above for primary myoblasts. After 24 h of treatment, MTS reagent was added according to the manufacturer's protocol (Promega, Madison, WI) and absorbance at 492 nm was read 2 h later.

Osteogenic differentiation and Alizarin Red S (ARS) staining was performed as described previously (Zhang et al., 2008). In brief, enriched BMSCs were plated in 24 well plates at a density of 5000 cells/cm^2 in proliferation medium and allowed to attach for 24 h. Cells were then treated with osteogenic induction medium (DMEM) supplemented with 5% FBS, 0.25 mM ascorbic acid, 0.1 μM dexamethasone, and 10 mM β glycerophosphate (all from Sigma Aldrich) for 14 d. For ARS staining, differentiated BMSCs were washed with PBS and fixed in 3% paraformaldehyde (Sigma Aldrich) for 30 min. Cells were stained with 40 mM ARS pH 4.1 (Sigma Aldrich) for 15 min followed by washing with excess dH$_2$O. Stained plates were scanned for visualization. For quantitative destaining, 10% acetic acid was added for 30 min. Samples were transferred to a 1.5 ml microcentrifuge tube, overlaid with mineral oil (Sigma Aldrich), heated to 85 °C for 10 min, and transferred to ice for 5 min. Following centrifugation at 14,000 rpm for 15 min, superna tants were removed and neutralized with 10% ammonium hydrox ide. Aliquots were transferred to a 96 well plate and absorbance was read at 405 nm (Gregory et al., 2004). Expression of osteogenic
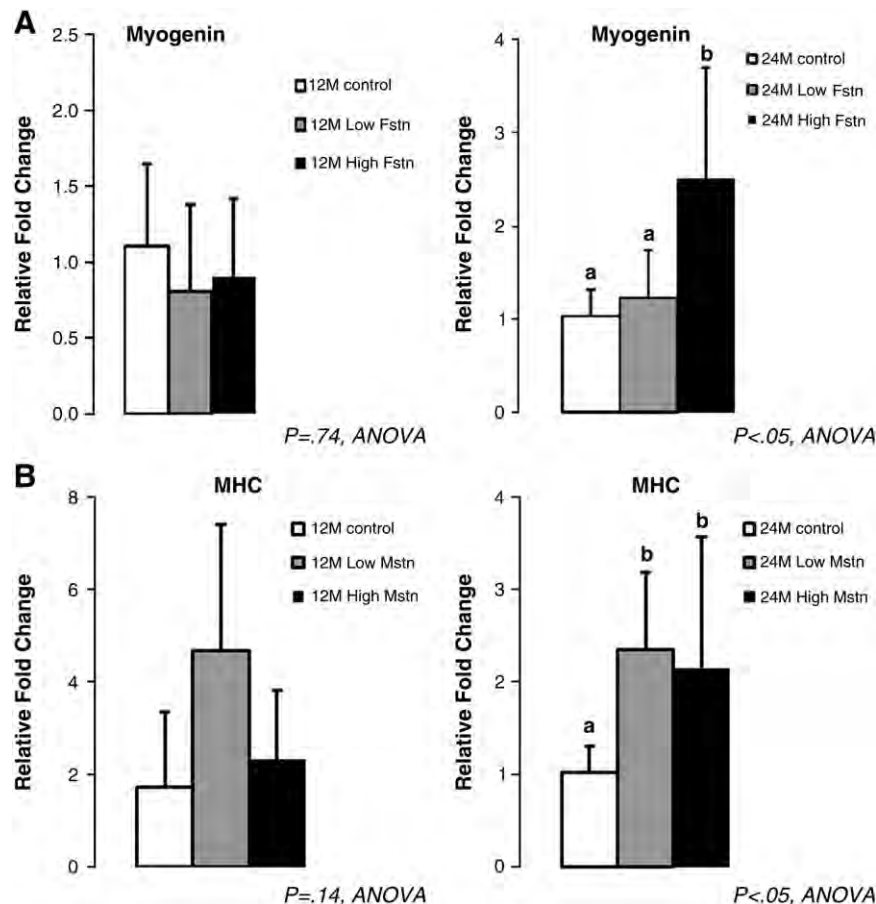

Fig. 3. Real-time PCR data for primary myoblast expression of myogenin (A) and myosin heavy chain (B) in response to treatment with follistatin (Fstn, top) and myostatin (Mstn, bottom). Means with different superscripts differ significantly from one another (P<.05). Error bars represent one standard deviation and sample size includes four-six replicates per group.
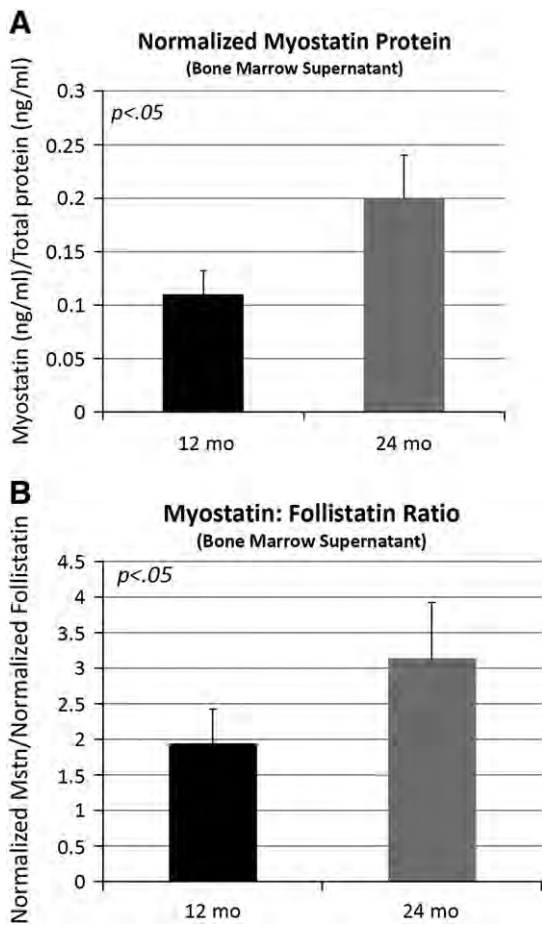
Fig. 4. Myostatin protein normalized to total protein (A) and the ratio of normalized myostatin to follistatin (B) in bone marrow supernatants from mice 12 months of age (12 mo) and 24 months of age (24 mo). Error bars represent one standard deviation and sample size includes six replicates per group.

genes was assessed using primers for bone morphogenetic protein 2 (BMP 2) and osteocalcin (OCN) shown in Table 1.

2.5. Statistical analysis

Two factor ANOVAs were performed with treatment and age as the two factors. Fisher's least significant difference (LSD) test was used for post hoc, pairwise comparisons. All statistical analyses were performed using SYSTAT® software.

3. Results

3.1. Protein levels of activin A, myostatin, and follistatin in skeletal muscle

ANOVAs performed on activin A and follistatin normalized for total protein, and the ratio of normalized activin A: follistatin, revealed no significant changes with age in either the soleus or extensor digitorum longus muscles. Levels of normalized myostatin showed a slight but non significant decrease in the EDL with age (Fig. 1A), whereas SOL showed a significant increase in normalized myostatin with age (Fig. 1A). Likewise, the ratio of normalized myostatin: follistatin showed a significant (~40%) decrease with age in EDL (Fig. 1B), but a significant (~four fold) increase with age in SOL (Fig. 1B). Two factor ANOVA with age (12 or 24 mo) and muscle (EDL or SOL) as the two factors showed significant age∗muscle interaction effects for both normalized myostatin (p<.05) and the

myostatin:follistatin ratio (p<.01), with myostatin levels decreasing with age in the EDL but increasing with age in SOL.

3.2. Effects of activin A, myostatin, and follistatin on the proliferation and differentiation of primary myoblasts

Overall there was a significant (P<.01) age effect for both myostatin and follistatin treatment, in which young cells were more proliferative in response to treatment than older cells. ANOVAs showed significant treatment effects for activin A, myostatin, and follistatin (Fig. 2A). Activin A produced a significant dose response increase in proliferation in myoblasts from younger mice but not older mice; however, the treatment∗age interaction was not significant. Follistatin also increased proliferation in dose response manner in young myoblasts, but the effect was attenuated in myoblasts from older mice (Fig. 2B). There was a significant (p=.001) treatment∗age interaction effect for follistatin treatment where the treatment effect, approximately two fold at the highest dose, was much greater in younger myoblasts compared to older cells. Myostatin treatment also increased proliferation in young myoblasts compared to untreated cells, whereas in older myoblasts myostatin decreased proliferation at the low dose (Fig. 2C). There was also a significant (p<.001) treatment∗age interaction effect for myostatin, where myostatin treatment increased proliferation in young myoblasts but decreased proliferation in the older cells.

RT PCR data revealed no marked changes in the expression of differentiation markers MyoD, myogenin, or myosin heavy chain (MHC) with Activin A treatment. MyoD and MHC expression were also unaffected by follistatin treatment of young and aged myoblasts; however, follistatin stimulated a significant increase in myogenin expression in aged but not young myoblasts (Fig. 3A). ANOVAs demonstrated a significant age effect for myogenin expression (p<.05) with older cells generally showing higher levels of myogenin expression, and there was also a significant treatment∗age interaction for myogenin expression. Myostatin treatment had no effect on MyoD or myogenin expression in either young or aged myoblasts, but myostatin did produce a significant increase in MHC expression in aged but not young myoblasts (Fig. 3B).

3.3. Protein levels of activin A, myostatin, and follistatin in bone marrow supernatants

Comparisons of normalized protein levels obtained from bone marrow supernatant revealed no significant differences between older and young mice for activin A, follistatin, or the activin A: follistatin ratio. Normalized myostatin is significantly (~80%) increased in mouse bone marrow with increasing age (Fig. 4A), as is the ratio of normalized myostatin: follistatin (+60%; Fig. 4B).

3.4. Effects of activin A, myostatin, and follistatin on the proliferation and differentiation of primary bone marrow stromal cells (BMSCs)

ANOVAs showed a significant (P<.001) age effect in each treatment group, with younger BMSCs overall having higher values for proliferation than older BMSCs, irrespective of the treatment (Fig. 5). Proliferation assays showed no effects of activin treatment on either young or old BMSCs (Fig. 5A). Two factor ANOVA revealed a significant treatment∗age interaction for follistatin, with follistatin having no impact on proliferation in older BMSCs but reducing proliferation in young BMSCs (Fig. 5B). Myostatin significantly decreased BMSC proliferation in both young cells (−15%) and aged cells (−40%); however, this effect was greater in the aged cells, and this treatment∗age interaction was also significant (P<.05) for myostatin (Fig. 5C).

Differentiation assays using alizarin red staining to detect mineralization revealed that activin treatment significantly increased mineralization of young and older BMSCs (Fig. 6). The effect of age on mineralization was significant (P<.001) for activin, with the
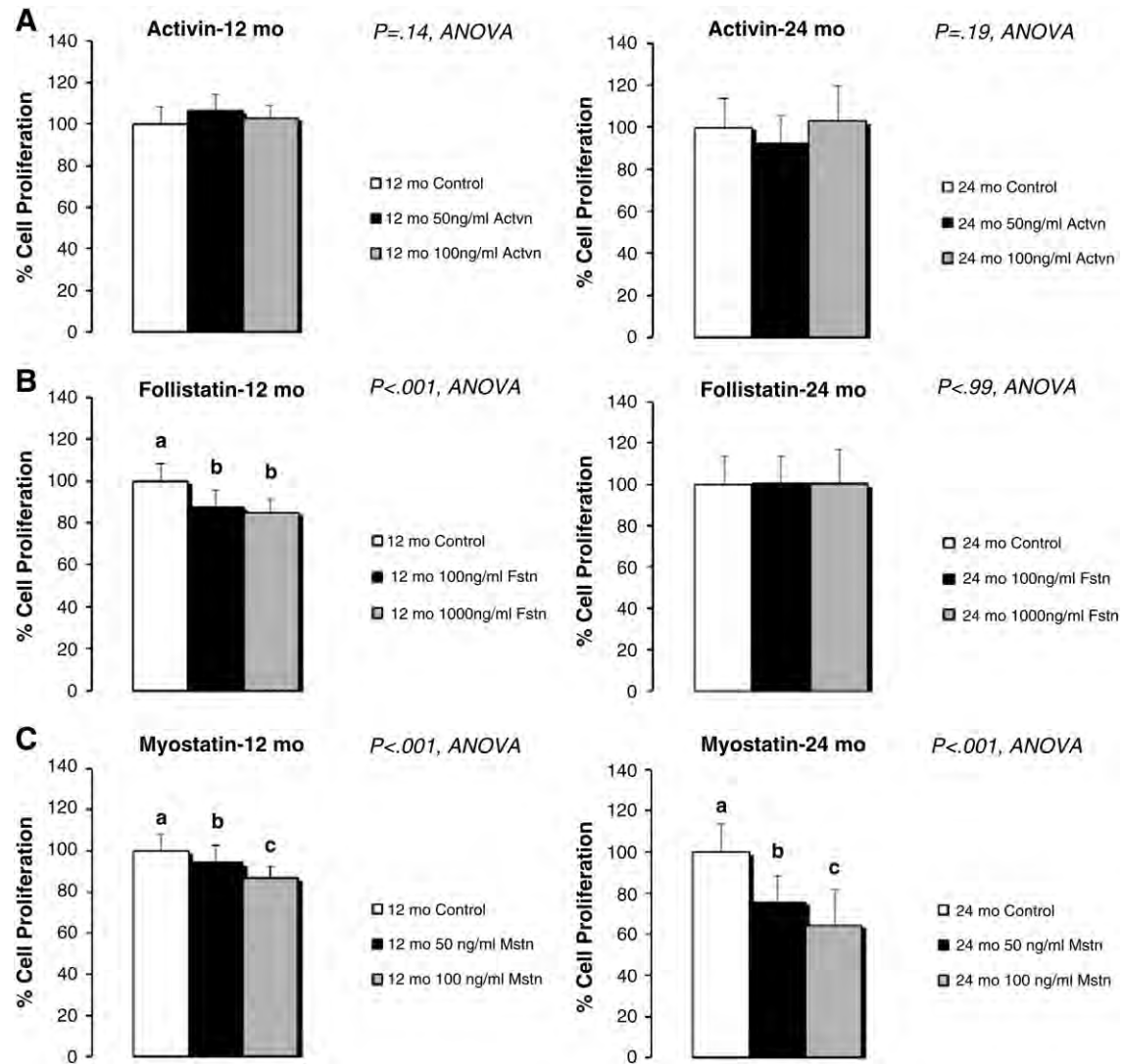
Fig. 5. Results of proliferation assays following treatment of primary bone marrow stromal cells (BMSCs) with activin A (A; activin), follistatin (B; Fstn), and myostatin (C; Mstn). Means with different superscripts differ significantly from one another (P<.05). Error bars represent one standard deviation and sample size includes eight replicates per group.

younger cells consistently showing greater mineralization in response to treatment. Treatment effects were less pronounced in aged BMSCs, with the low dose of activin increasing alizarin red staining but the other doses showing no significant effect (Fig. 6C,D). The treatment∗age interaction was significant (P<.01) for activin using two factor ANOVA. In the myostatin experiments, the higher dose of myostatin significantly decreased mineralization in younger cells but not in older cells, and the treatment∗age interaction was also significant for myostatin (P<.01). Finally, follistatin treatment did not significantly affect mineralization either young or older BMSCS (Fig. 6).

4. Discussion

The ELISA assays using muscle and bone samples from aged mice provided results that were unexpected for two reasons. First, it is well known that fast twitch (type II) muscle fibers atrophy with age (Holloszy et al., 1991) and a number of studies have implicated myostatin in muscle atrophy (McKay et al., 2012). In addition, Hennebry et al. (2009) found that mice lacking myostatin showed an increase in type I fibers and decrease in type II fibers, and blocking myostatin function in vivo using a myostatin antibody has been shown to increases type II fiber size in aging mice (Murphy et al.,

2010). The fact that we observed a decline in myostatin levels with age in the predominantly fast twitch extensor digitorum longus muscle of mice, but a relative increase in myostatin levels in the slow twitch (type I) soleus, was therefore not anticipated. Nevertheless, it has been shown that mice lacking myostatin lose the same percentage of muscle mass with age as normal mice (Morissette et al., 2009), and muscles composed of different fiber types are all larger in aged myostatin deficient mice compared to those of same aged wild type mice (Jackson et al., 2012). Given that both myostatin levels and the myostatin:follistatin ratio increased with age in soleus muscles of mice compared to extensor digitorum longus, myostatin inhibitors may have a greater impact on preserving muscle mass with age in those muscles composed predominantly of slow rather than fast twitch fibers.

The second in vivo finding that was also unexpected was the significant rise in myostatin protein with age in bone marrow supernatants. This was not expected because myostatin is primarily secreted by muscle and not by osteoblasts or marrow stromal cells. Yarasheski et al. (2002) did, however, find that serum myostatin increased with age in older women, and Szulc et al. (2012) found that serum myostatin increased in men until age 57 and then declined. The rise in bone marrow myostatin that we observed with age in mice likely reflects an increase
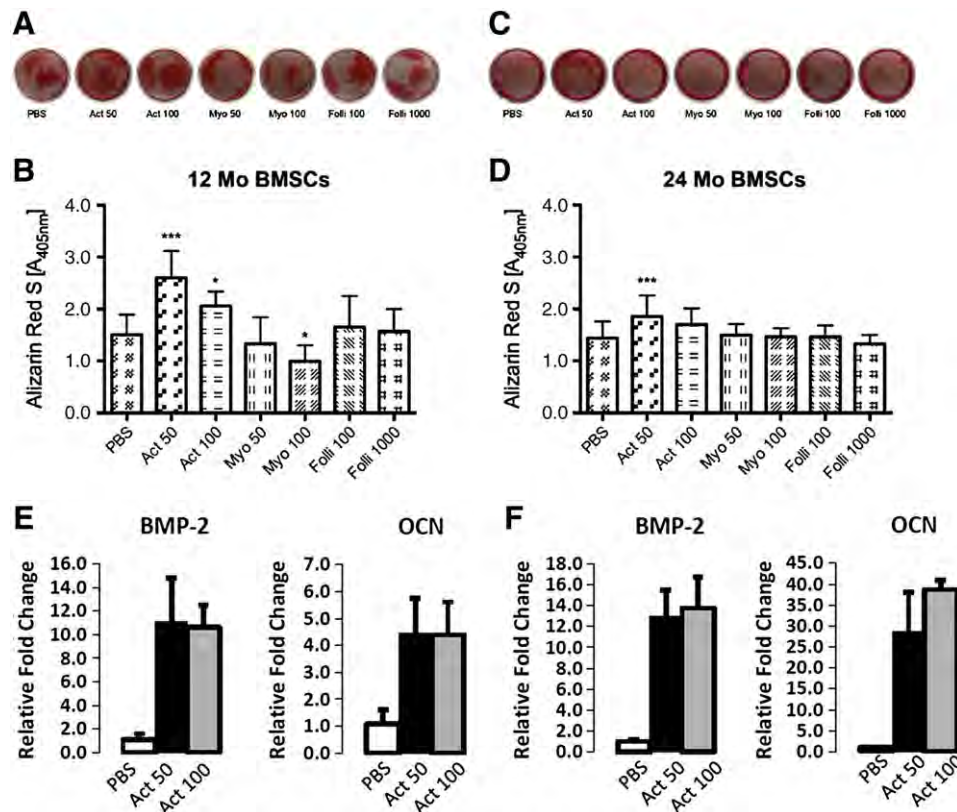
Fig. 6. Alizarin red staining of bone marrow stromal cells cultured in osteogenic conditions (A–D) and PCR data for osteogenic genes (E–F) from BMSCs treated with activin A. Images of wells (A, C) and quantification of staining (B, D) in BMSCs from young mice (A, B; 12 mo BMSCs) and older mice (C, D; 24 mo BMSCs) treated with Activin A (Act), Myostatin (Myo) or Follistatin (Folli) at 50 ng/ml (50), 100 ng/ml (100) or 1000 ng/ml (1000). ***P<.001 and *P<.05 relative to same-aged PBS controls. Error bars represent one standard deviation and sample size includes sixteen replicates per group for panels A–D. PCR data show elevated expression of osteogenic genes BMP-2 and osteocalcin (OCN) in activin-treated cells from mice 12 months of age (E) and mice 24 months of age (F). Error bars represent one standard deviation and four replicates are included per treatment group.

in circulating myostatin levels with age. The elevated concentrations of myostatin in the bone marrow microenvironment of aged mice is significant given the findings discussed below regarding the effects of myostatin on proliferation of bone marrow stromal cells. Moreover, while myostatin was found to be most highly expressed in skeletal muscle progenitors early in development (McPherron et al., 1997), data presented here and elsewhere suggest that myostatin levels may rise again later in life. Thus, the interplay among activin A, myostatin, and follistatin is dynamic and changes across the aging spectrum.

The cell proliferation experiments yielded findings that were consistent with those of previous studies with regard to the effects of age and treatment. First, in both primary myoblasts and BMSCs, age effects were significant regardless of treatment. That is, aged myoblasts and BMSCs were less proliferative overall in response to treatment whether the cells were exposed to activin A, myostatin, or follistatin. This does not appear to be related to expression of ActRIIB, since we have observed using PCR (data not shown) that ActRIIB expression does not differ significantly either between muscle and bone cells of mice at similar ages, or between musculoskeletal tissues of young versus aged mice. Second, in the case of primary myoblasts, all of the ligands generally increased proliferation of young myoblasts at lower doses. Activin A was previously found to suppress embryonic muscle development in chicks (He et al., 2005), and to inhibit proliferation of stem cells derived from adult muscle (Nomura et al., 2008). Myostatin has also been shown previously to be a potent inhibitor of myoblast proliferation (McFarlane et al., 2011), and while myostatin increased proliferation of young myoblasts it decreased proliferation of aged myoblasts. The effects of these ligands on proliferation were quite different in BMSCs, where follistatin and myostatin both suppressed

proliferation of young BMSCs, and myostatin significantly suppressed the proliferation of aged BMSCs. These data are consistent with our previous findings showing that BMSCs of mice lacking myostatin were more proliferative in vitro than those from normal mice (Elkasrawy et al., 2011). Probably the most important observation from these in vitro experiments is that, in the case of both aged myoblasts and aged BMSCs, myostatin consistently suppressed cell proliferation. Aged muscle precursors have an impaired capacity for proliferation (Conboy et al., 2003; Machida and Booth, 2004), as do aged BMSCs (Kretlow et al., 2008). Blocking myostatin function in aged animals may therefore have significant potential for improving the repair and regeneration of both muscle and bone by improving the proliferative capacity of both myo and osteo progenitor cells.

Findings from our differentiation assays are generally consistent with the idea that myostatin inhibits proliferation and promotes terminal differentiation of aged myoblasts, as myostatin treatment increased myosin heavy chain expression in aged myoblasts. The osteoblast differentiation and mineralization assays revealed that while activin A increased mineralization, myostatin exposure suppressed mineralization in vitro (Fig. 6). These experiments where BMSCs were treated with myostatin are also consistent with our previous studies using BMSCs from myostatin deficient mice, which showed that Mstn$^{-/-}$ BMSCs had an increased capacity for mineralization in vitro and impaired potential for adipocyte differentiation (Hamrick et al., 2007; Kellum et al., 2009). The activin A differentiation experiments with BMSCs differ somewhat from those of Eijken et al. (2007) who found that activin A treatment strongly inhibited mineralization in osteoblast cultures, whereas follistatin increased mineralization. Our data, on the other hand, showed that osteogenic culture of BMSCs in the presence

of activin A increased mineralization whereas follistatin had no effect on mineralization in either young or old BMSCs. It is likely that our findings differ from those of Eijken et al. (2007) because our experiments exam ined the effects of activin A and follistatin on the differentiation of osteoprogenitors, whereas Eijken et al. treated differentiated osteo blasts. It is therefore important to acknowledge here that, while our study examined the proliferation and differentiation of progenitor cell populations, ligands of the activin A myostatin follistatin system may have different effects on target cells at different stages of development, differentiation, and maturation.

5. Conclusions

Our experiments using aged mice as a model system for investi gating the role of activin A, myostatin, and follistatin in musculoskel etal aging suggest that these ligands are likely to be involved in the altered capacity for tissue formation and repair that is observed in aged rodents and humans. Specifically, primary myoblasts and bone marrow stromal cells of aged animals were found to be less proliferative than those of younger animals in response to most of the treatments utilized. Myostatin levels in bone marrow and in soleus muscles increased with age in mice, and myostatin treatment suppressed proliferation of both aged myoblasts and aged BMSCs. Myostatin also inhibited mineralization of BMSCs under osteogenic cul ture conditions. Together these data suggest that targeting myostatin in aged animals may improve the proliferative capacity of muscle and bone progenitor cells, perhaps enhancing the potential for muscle and bone repair and regeneration in the therapeutic setting.

Acknowledgments

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A Myostatin Inhibitor (Propeptide-Fc) Increases Muscle Mass and Muscle Fiber Size in Aged Mice but Does not Increase Bone Density or Bone Strength

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Abstract

Loss of muscle and bone mass with age are significant contributors to falls and fractures among the elderly. Myostatin deficiency is associated with increased muscle mass in mice, dogs, cows, sheep and humans, and mice lacking myostatin have been observed to show increased bone density in the limb, spine, and jaw. Transgenic overexpression of myostatin propeptide, which binds to and inhibits the active myostatin ligand, also increases muscle mass and bone density in mice. We therefore sought to test the hypothesis that in vivo inhibition of myostatin using an injectable myostatin propeptide (GDF8 propeptide-Fc) would increase both muscle mass and bone density in aged (24 mo) mice. Mice were injected weekly (20 mg/kg body weight) with recombinant myostatin propeptide-Fc (PRO) or vehicle (VEH; saline) for four weeks. There was no difference in body weight between the two groups at the end of the treatment period, but PRO treatment significantly increased mass of the tibialis anterior muscle (+7%) and increased muscle fiber diameter of the extensor digitorum longus (+16%) and soleus (+6%) muscles compared to VEH treatment. Bone volume relative to total volume (BV/TV) of the femur calculated by microCT did not differ significantly between PRO- and VEH-treated mice, and ultimate force (Fu), stiffness (S), toughness (U) measured from three-point bending tests also did not differ significantly between groups. Histomorphometric assays also revealed no differences in bone formation or resorption in response to PRO treatment. These data suggest that while developmental perturbation of myostatin signaling through either gene knockout or transgenic inhibition may alter both muscle and bone mass in mice, pharmacological inhibition of myostatin in aged mice has a more pronounced effect on skeletal muscle than on bone.

Keywords

sarcopenia; osteoporosis; fractures; anabolic therapy

1. Introduction

Globally, the size of the aging population is increasing rapidly, and as a corollary the prevalence of age-related musculoskeletal disorders such as osteoarthritis, sarcopenia, and

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osteoporosis is also increasing (Sanchez-Riera et al., 2010). A primary contributor to the morbidity and mortality associated with aging is an increased frequency of falls, and falls are often accompanied by bone fractures. Indeed, falls are the primary factor in more than 90% of bone fractures (Jarvinen et al., 2008). In many cases bone fractures limit subsequent capacity for normal daily activities, ambulation, and independence, ultimately leading to assisted living situations which can be financially burdensome. The disability following a fall and fracture also contributes directly to an increase in comorities such as respiratory infections, which in turn contribute to greater overall age-related mortality (Bertram et al., 2011).

The growth, development, and aging of muscle and bone are closely linked. Pediatric gains in bone mass are normally preceded by gains in muscle mass, and loss of muscle mass with age typically precedes peak rates of loss in bone density (Hamrick et al., 2010a). The close coupling of muscle and bone across the lifespan therefore suggests that changes in one tissue may be mechanistically linked with changes in another. Indeed, there are multiple mechanisms linking the two tissues such as mechanical loading, muscle-derived trophic factors (myokines), as well as systemic factors such as sex steroids and growth factors that have anabolic effects on both muscle and bone. The functional and perhaps molecular integration between the two tissues therefore suggests that therapeutic strategies targeting one particular tissue may have positive effects on the other, or that certain pharmacologic approaches (e.g., androgen-receptor modulators, vitamin D receptor agonists) could positively impact both tissues at once (Hamrick 2010, 2011, 2012).

Given the very close linkages between muscle and bone referenced above, we sought to test the hypothesis that pharmacologic inhibition of myostatin (GDF-8) could increase both muscle and bone mass in an aged animal model. Our rationale for pursuing this hypothesis is based on several key observations. The first is that mice lacking myostatin show increased muscle mass as well as increased bone density in the spine, limb, and jaw (Elkasrawy et al., 2010). The second is that recent studies have demonstrated that a myostatin antibody (LeBrasseur et al., 2009; Murphy et al., 2010) and a decoy myostatin receptor (Chiu et al., 2013) can increase muscle mass in aged mice. The decoy receptor (ActR-IIB) was also found to increase bone formation and bone density (Chiu et al., 2013). These findings suggest that therapeutic modulation of myostatin in vivo may be an effective strategy for preserving muscle and bone mass with age, and so we employed a mouse model to evaluate this hypothesis. Specifically, we have previously shown that C57BL6 mice lose significant muscle mass, bone density, and bone strength with age, such that mice 24 months of age show a marked decrease in these measures compared to mice at 12 months of age (Hamrick et al., 2006a). This study utilizes myostatin propeptide treatment in aged (24 months) C57BL6 mice as an in vivo model for assessing the potential effects of myostatin inhibition on age associated muscle and bone loss. The propeptide fragment is utilized here because it has previously been shown to enhance muscle and bone repair in vivo, and binds the active myostatin ligand with high affinity (Bogdanovich et al., 2005; Hamrick et al, 2010b).

2. Materials & Methods

2.1 Production and validation of myostatin propeptide

The myostatin propeptide-Fc fusion protein was produced in CHO cells as described previously (Jiang et al., 2004). To measure the activity of myostatin and the efficacy of the myostatin propeptide a luciferase reporter gene assay was developed where the vector pGL3(CAGA)12 – neo was stably transfected into A204 (human rhabdomyosarcoma) cells. Addition of myostatin to the A204 CAGA cells, and the binding of myostatin to its receptors, initiates the Smad signal transduction pathway and activates the luciferase reporter gene. The level of activation is proportional to the luciferase activity and the linear

portion of the activity curve is in the ng/ml range (Fig. 1), which is what is expected for a protein in the TGFβ superfamily. The addition of the myostatin propeptide prevents the binding of myostatin to its receptors, and the IC50 for the propeptide is approximately 2.0 nM (Fig. 1).

2.2 Animals and treatment for aging study

C57BL6 mice were purchased from the aged rodent colony at the National Institute on Aging, National Institutes of Health (USA) at 22 months of age and delivered to Georgia Regents University, Augusta GA. Animals were allowed to acclimate for one week and were maintained at the Laboratory Animal Service Facility of Georgia Regents University. An earlier dose-response study was used to evaluate the efficacy of a myostatin propeptide in vivo (Hamrick et al., 2010b). Adult mice (5–6 mo.) were treated with the propeptide at 0, 10, 20, or 50 mg/kg at day 0, 5, and 10 and then sacrificed one week after the last treatment. Those data showed that propeptide treatment increased fore- and hindlimb muscle mass by 10% at the 10 mg/kg dose and increased muscle mass by more than 15% at the 20 mg/kg dose, but the 50 mg/kg dose did not increase muscle mass beyond the increase observed in the 20 mg/kg group (Hamrick et al., 2010b). The 20 mg/kg dose was therefore used in this study. Mice were divided into two treatment groups: a vehicle group (VEH; n=14) and a myostatin propeptide group (PRO; n=15). Mice received i.p. injections every five days for 25 days with a dosage of 20 mg/kg body weight at a volume of 0.2 ml. Myostatin propeptide [4.48mg/ml] was obtained from Pfizer Inc (Cambridge, MA, USA). Mice were given calcein i.p. injections to label actively mineralizing bone surfaces four days and 24 hours prior to sacrifice.

2.3 Tissue collection

Animals were euthanized by CO_2 overdose and thoracotomy following procedures approved by the Institutional Animal Care and Use Committee of Georgia Regents University. The extensor digitorum longus (EDL) and soleus (SOL) muscles from one limb were dissected out, cut in half and embedded in OCT for cryostat sectioning and muscle fiber size measurements. The tibialis anterior from one limb was dissected out, weighed, snap frozen and homogenized for RT-PCR of the following myogenic markers: myostatin, Mafbx, Murf 1, MHC, and IGF-1. The right tibias were disarticulated and fixed in 70% ethanol for μCT and plastic sectioning while the left tibias were stored damp at minus 20°C for biomechanical testing followed by RT-PCR for bone formation and osteoblast differentiation markers Runx2, Osx and BMP-2. The femora were fixed in 4% paraformaldehyde, decalcified and embedded in paraffin for sectioning and stained for osteoclasts (TRAP kit from Sigma 386A-1KT) and osteoblasts (Celestine blue/van Geison's).

2.4 Bone Histomorphometry

Osteoblast and osteoclast counts were performed as previously described (Wenger et al., 2010) on 4–5 μm sections after the specimens were decalcified in 4% EDTA for 1 week, dehydrated, cleared in xylene, then embedded in paraffin. Osteoblasts were counted on sections stained using von Giessen, and osteoclasts counted on sections stained for tartrate-resistant acid phosphatase (TRAP) activity. Standardized peripheral locations from the metaphysis were measured in a fixed region of interest. Mineralizing surfaces were measured from calcein-labeled, undecalcified bone sections. Tibias fixed in 70% ethanol were dehydrated and embedded in methyl methacrylate and sectioned in the horizontal (transverse) plane. Sections were viewed using a Zeiss Axioplan2 fluorescent microscope and captured using a SPOT® digital camera to image labeled bone surfaces. Forming surface was calculated as the percentage of non-eroded, single-labeled surface/total surface $\times$ 100 (MS/BS).

2.5 Biomechanical testing and micro-computed tomography (uCT)

Left tibias were stored damp at −20°C before being allowed to thaw at room temperature in PBS for 1 hr. Specimens were tested in three-point bending using a Vitrodyne V1000 Material Testing system as described previously (Hamrick et al., 2006b, 2008). Tibias were mounted antero-posteriorly on stainless steel fixtures 5mm apart, approximately 2.5 mm either side of center. Testing was linear displacement control with a displacement speed of 10 μm/sec using a Transducer Techniques 5 kg load cell. Structural, or extrinsic, properties including ultimate force (Fu; height of curve) and stiffness (S; slope of curve) were calculated from load-displacement curves. MicroCT images of the right tibia were scanned using Bruker Skyscan1174 compact micro-CT (Belgium), software version 1.5 (build 9) using NRecon version 1.6.4.8 for reconstructed images.

2.6 PCR and Western blotting

Total RNA was extracted using Trizol and cDNA was synthesized using Quantitect reverse transcription kit (catalog no. 205310; Qiagen). Expression was analyzed quantitatively by means of the Quantitect SYBR Green PCR kit (catalog no. 204143, Qiagen), and QuantiTect Primer Assays. We used specific primers provided by QuantiTect Primer Assays for Myostatin, Murf1, MaFbx, MHC, IGF-1, Runx2, Osx, BMP-2 and 18S, GAPDH and β-actin (internal controls; Table 1). Half of each extensor digitorum longus muscle was snap-frozen in liquid nitrogen for Western blotting. Each muscle was placed in phosphate buffered saline (PBS) and subjected to homogenization using Fisherbrand Tissuemiser® rotary homogenizer until large pieces of muscle were no longer visible. Samples were subjected to two freeze–thaw cycles to disrupt the plasma membrane then centrifuged briefly. Protein concentrations were measured using a commercial BCA reagent (Pierce, Rockford, IL) to ensure equal loading. 30 μg of proteins from whole tissue lysates were mixed 1:1 with 2× sample buffer and then applied to 4–20 % polyacrylamide gels. Samples were electrophoretically separated and transferred to nitrocellulose membrane (Invitrogen, Carlsbad, CA). The membranes were incubated with specific primary antibodies MURF1 (Abcam cat. 77577) or MAFbx (Santa Cruz Biotech cat. 166806) and then incubated with anti-mouse or anti-rabbit peroxidase-conjugated secondary antibodies (Santa Cruz, CA). After the incubation, the membranes were washed three times for 15 min each with 1× TTBS solution and then incubated with 1 ml of chemiluminescence reagent (Invitrogen). The protein bands were visualized using X-ray films (Fisher Scientific, Rochester, NY).

2.7 Statistical analysis

Single-factor ANOVA with treatment group as the factor was used to for pairwise comparisons of morphometric and histomorphometric parameters. For analysis of gene expression data, the control genes of 18S and Actin were averaged to obtain an average control gene for muscle tissue while GAPDH was used as the control gene for bone. Difference in control gene Ct expression between GDF-8 and vehicle was assessed using a two-sample t-test. Delta Ct values for each treatment group were calculated as $\Delta Ct = Ct_{Target\ gene} - Ct_{Control\ gene}$. The difference in ΔCt expression between GDF-8 and vehicle was assessed using a two-sample t-test. The magnitude of the difference between the groups was estimated using deltadelta Ct values for each target gene and these were calculated as $\Delta\Delta Ct = \Delta Ct_{GDF-8} - \Delta Ct_{vehicle}$ and fold change was calculated as 2 to the power $-\Delta\Delta Ct$. SAS® version 9.3 (SAS Institute, Inc., Cary, NC) was used for all analyses and alpha=0.05 was used to determine statistical significance.

3. Results

3.1 Myostatin propeptide increases muscle mass and fiber size in aged mice

Body weight of the vehicle- and propeptide-treated animals was similar at the end of the study (Fig 2A). Each treatment group did, however, lose some weight over the treatment period but this was less dramatic for the treated animals, such that their decrease in body weight from day 0 to day 25 was significantly less than that of the vehicle-treated mice (Fig. 2B). Muscle mass of the tibialis anterior was significantly increased in the treated mice, both absolutely (Fig. 3A) and relative to body weight (Fig. 3B). Fiber size of the predominantly fast-twitch extensor digitorum longus (EDL) muscle was also significantly increased by more than 15% in the treated mice (Fig. 3C,D), whereas the increase in muscle fiber size in the predominantly slow-twitch soleus (SOL) muscle was also increased significantly (Fig. 3E) but by a lesser magnitude (~5%). Propeptide treatment produced a slight but non-significant increase in the expression of myostatin itself, as well as expression of myosin heavy chain and IGF-1 (Fig. 4A). Surprisingly, expression of the ubiquitin ligases Murf1 and Mafbx was significantly increased with propeptide treatment (Fig. 4A), and the PCR data were further validated by Western blot (Fig. 4B).

3.2 Myostatin inhibitor does not alter bone formation or bone strength in aged mice

MicroCT data from the tibia show that bone mineral density is actually slightly higher (3%) in the tibias of vehicle-treated mice (Table 2), but other parameters such as bone volume relative to total volume, trabecular number, and trabecular thickness are similar between the two groups (Table 2). Likewise, three-point bending tests of tibias show that ultimate force, stiffness, and toughness (energy to fracture) are also similar between the vehicle- and propeptide-treated mice (Table 2). Bone histomorphometry data reveal that osteoblast and osteoclast numbers do not differ between the experimental groups (Table 3). Fluorochrome labeling showed double-labels in only three mice from each group, and so single-labeled surfaces were compared. Actively mineralizing surfaces were also similar between the two groups of mice (Table 3). Gene expression data show no significant differences in the expression of osteogenic genes Osx or Runx2 with propeptide treatment, however the expression of BMP-2 is increased in animals receiving the propeptide (Fig. 4C).

4. Discussion

Pharmacological inhibition of myostatin has, to date, been pursued using a variety of in vivo approaches. These include utilization of myostatin-specific antibodies (Bogdanovich et al., 2002; Wagner et al., 2008; LeBrasseur et al., 2009; Murphy et al., 2010), a decoy myostatin receptor (ActRIIB-Fc; Lee et al., 2005; Bialek et al. 2008; Borgstein et al., 2009; Chiu et al., 2013), and myostatin propeptide (Bogdanovich et al., 2005; Hamrick et al., 2010b). Published data now exist in which each of these therapies has been evaluated in aged rodents, so that some comparison of the different approaches can be undertaken. Our data using a myostatin propeptide are consistent with data from studies using myostatin antibodies, where these myostatin inhibitors were found to have significant, positive effects on muscle mass, fiber size, and muscle force production. Specifically, LeBrasseur et al. (2009) used a slightly higher dose (25 mg/kg) than we used in our study (20 mg/kg), but also used weekly injections of a myostatin inhibitor (PF-354 antibody) over a period of 4 weeks in mice 24 months of age. They too found a moderate (<10%) in muscle mass and a significant increase in muscle mass relative to body weight (+12–17%), as we did for the tibialis anterior muscle relative to body weight (+~15%). Murphy et al. (2010), like LeBrasseur et al. (2009), used weekly doses of the PF-354 antibody but used a lower dose (10 mg/kg) for a longer treatment period—14 weeks of treatment starting in mice aged 18 months. These authors found increases in overall muscle mass (<10%) in the gastrocnemius

and quadriceps of the aged mice following 14 weeks of treatment, and a significant increase in (+12%) in muscle fiber cross-sectional area of the tibialis anterior muscle, that were similar in magnitude to the changes we observed with propeptide treatment. Together, these studies using myostatin antibodies and our study using the myostatin propeptide show similar increases in muscle fiber size and muscle mass using either a low dose (10 mg/kg) over a longer (14 week) treatment period, or a higher dose (20–25 mg/kg) over a shorter treatment period (4 weeks).

Data from in vivo studies using either the myostatin antibody or the propeptide differ in two important ways from those utilizing the decoy myostatin receptor (ActRIIB-Fc). First, a 10 mg/kg dose of ActRIIB-Fc administered twice weekly for four weeks increased tibialis anterior mass by 30% and quadriceps mass by 25% (Chiu et al., 2013). These increases in muscle mass are much greater than those observed with either the myostatin antibody or propeptide, which as noted above generated increases in total muscle mass of <10%. It is possible that these differences could be due to the more frequent administration of the ActRIIB-Fc, but the ActRIIB-Fc dose is much lower than that used in either our study or the study by LeBrasseur et al. (2009), suggesting that the ActRIIB-Fc is a more potent molecule for increasing muscle mass in aged mice. The reason for the greater potency of the ActRIIB-Fc for increasing muscle mass is likely because this molecule can bind several ligands in addition to myostatin, including activin, BMP-3, BMP-7, BMP-9, BMP-10, and GDF-11 (Souza et al., 2008). Some of these molecules, such as activin, are also likely to play a role in regulating muscle mass, which is further indicated by the fact that ActRIIB-Fc treatment can increase muscle mass in mice that lack myostatin altogether (Lee et al., 2005). The second way in which our data differ from those using ActRIIB-Fc is related to the effects on bone. ActRIIB-Fc treatment was previously documented to increase bone formation and bone mass in young, growing mice (Bialek et al., 2008; Yan et al., 2008), and the data from Chiu et al. (2013) are consistent with this earlier report in showing that ActRIIB-Fc increases bone density and serum markers of bone formation in aged mice after just 30 days of treatment. In contrast, our data revealed no bone effects with myostatin propeptide treatment. These data may indicate that, as proposed by Chiu et al. (2013), the anabolic effects of ActRIIB-Fc on bone are due to antagonizing effects on ligands other than myostatin, such as various BMPs or activin.

Previous work in our lab showed that myostatin can inhibit the proliferation of aged bone marrow stromal cells (Bowser et al., 2013), that bone marrow stromal cells from mice lacking myostatin show increased proliferation (Elkasrawy et al., 2011), and that myostatin can inhibit chondrogenesis in vivo and in vitro (Elksrawy et al., 2012). These data may at least in part explain the increased fracture callus size following osteotomy in mice lacking myostatin (Kellum et al., 2009), and increased fracture callus bone volume in mice treated with myostatin propeptide following osteotomy (Hamrick et al., 2010). That is, myostatin seems to play a key role in musculoskeletal injury repair, one in which myostatin secretion from muscle is elevated following muscle damage, and then mediates the repair response in adjacent bone by modulating progenitor cell proliferation (Elkawrawy et al., 2012). On the other hand, myostatin appears to have a more limited role in mature, intact bone. This is indicated by the fact that myostatin itself is not expressed at a significant level by osteoblasts (Digirolamo et al., 2011), and that myostatin inhibition via propeptide treatment in adult mice does not significantly alter osteoblast number, mineralizing surfaces, or bone strength (Tables 1 and 2). Thus, therapeutic targeting of myostatin specifically via antibody or propeptide treatment may have clinical application in the context of improving muscle mass alone, or improving the healing of muscle and bone following injury, but is not likely to have a significant impact on bone formation in the intact, aged animal. In contrast, the decoy myostatin receptor (ActRIIB-Fc) appears capable of increasing muscle mass, bone formation, and bone strength in aged rodents, suggesting that this molecule may have

potential clinical use for age-associated loss of both muscle and bone in the form of sarcopenia and osteoporosis.

Muscle and bone are closely associated spatially and in terms of structure and function during growth, development, and aging. Muscle in particular has been considered a driving force for bone modeling and remodeling, in that muscle is the primary source of mechanical stimuli for bone and bone tissue is thought to adapt its gross structure in response to muscle-derived stimuli. Thus, targeting muscle therapeutically is thought to be one approach for improving bone health, simply by enhancing the mechanical relationship between muscle and bone. On the other hand, a large portion of osteoporotic fractures do not occur in individuals with low bone density as measured by two-dimensional densitometry, and so fall prevention alone may be another strategy for reducing falls and fall-associated morbidity and mortality in the elderly (Jarvinen et al., 2008). Behavioral interventions such as resistance exercise or nutritional interventions such as vitamin D supplementation (Girgis et al., 2013) may improve muscle strength and/or neuromuscular control and proprioception, perhaps reducing fall risk. The extent to which myostatin inhibition may augment such strategies remains relatively unexplored. Mice are relatively small in body weight and their bones are capable of withstanding loads many times their own body mass--for example it takes more than 2 kg of force to fracture the tibia of a 32 g mouse (Fig. 1, Table 2). Thus, increases in muscle mass in these small mammals may not significantly alter the mechanical environment of their bones. Additional studies in patient populations are needed to determine the extent to which therapeutic targeting of muscle alone via a myostatin antibody or propeptide, perhaps in conjunction with an exercise regimen, could reduce the incidence of bone fractures versus a molecule such as ActRIIB-Fc, that may potentially increase the mass and strength of both muscle and bone.

5. Conclusions

We tested the hypothesis that in vivo inhibition of myostatin using an injectable myostatin propeptide (GDF8 propeptide-Fc) would increase both muscle mass and bone density in aged (24 mo) mice. Our goal was to evaluate this potential therapeutic for its capacity to increase both muscle and bone mass in the setting of age-associated sarcopenia and osteoporosis. Mice were injected weekly (20 mg/kg body weight) with recombinant myostatin propeptide-Fc (PRO) or vehicle (VEH; saline) for four weeks. The data show that PRO treatment significantly increases muscle fiber size and muscle mass, both absolutely and relative to body weight. In contrast bone volume, bone strength, and histomorphometric parameters of bone formation and bone resorption were unchanged with PRO treatment. Our findings are consistent with previous studies utilizing a myostatin antibody in aged mice showing that targeting myostatin increases muscle fiber size and mass; however, our data differ from work utilizing a decoy myostatin receptor (ActRIIB-Fc) to inhibit myostatin function in that ActRIIB-Fc appears particularly effective at increasing bone density and bone formation whereas the propeptide does not. The anabolic effects of ActRIIB-Fc on aged bone are likely due to the ability of this molecule to antagonize other ligands besides myostatin, such as activin or bone morphogenetic proteins. Clinical trials evaluating the potential of these molecules to prevent falls and fractures are needed to determine the optimal approaches for reducing musculoskeletal diseases and complications in the elderly.

Acknowledgments

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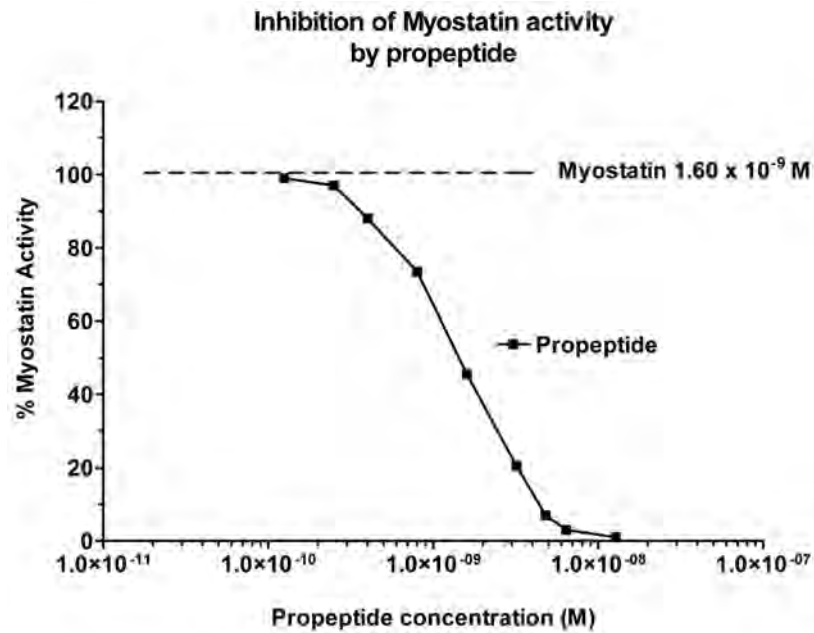
Inhibition of Myostatin activity
by propeptide

Figure 1.
Myostatin-induced luciferase activity declines significantly with increasing concentration of myostatin propeptide. The pGL3(CAGA)12 – neo reporter vector contains 12 CAGA boxes previously reported to be TGF-β-responsive elements (Dennler et al. (1998) EMBO J. 17:3091–3100), a neo resistance gene, and the basic luciferase reporter plasmid pGL3 (Promega Corporation).
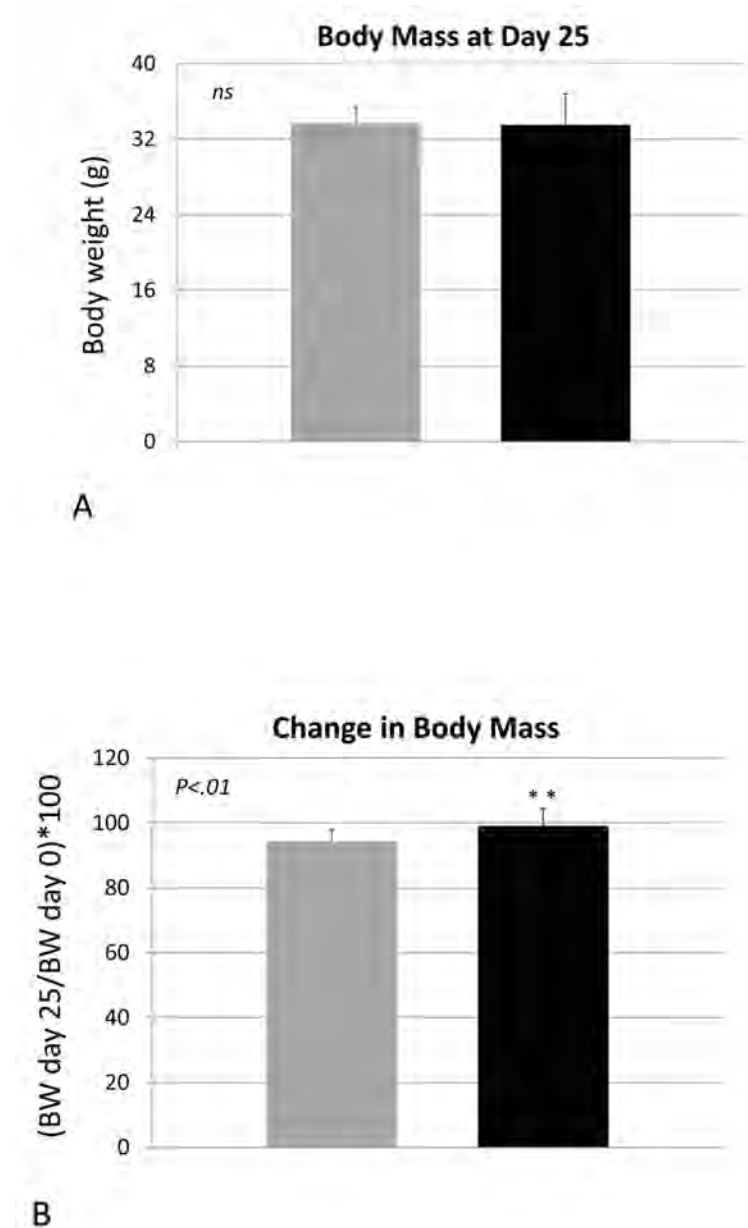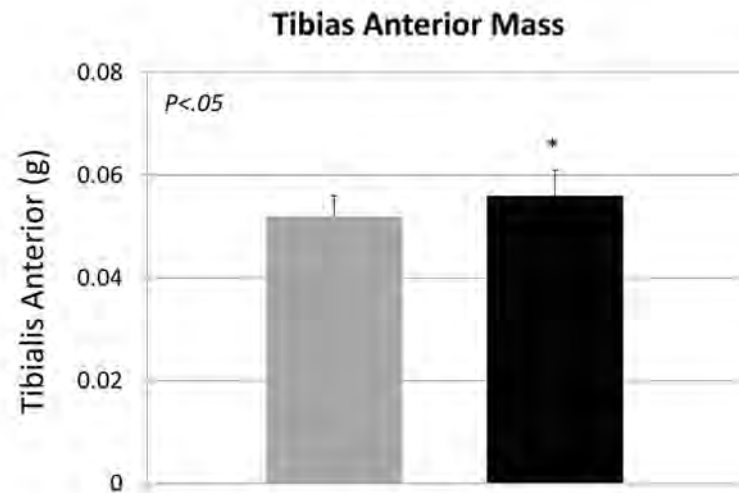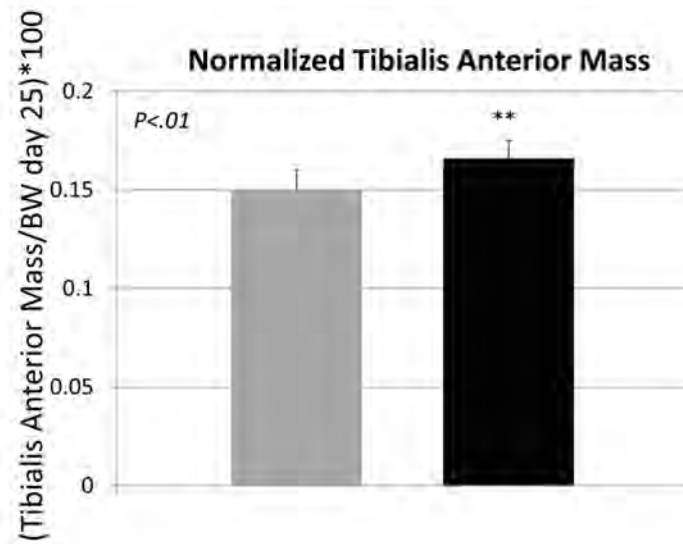
Figure 2.
Body mass (A) and change in body weight (B) for animals treated with either saline (VEH) or myostatin propeptide (PRO) weekly for over four weeks.
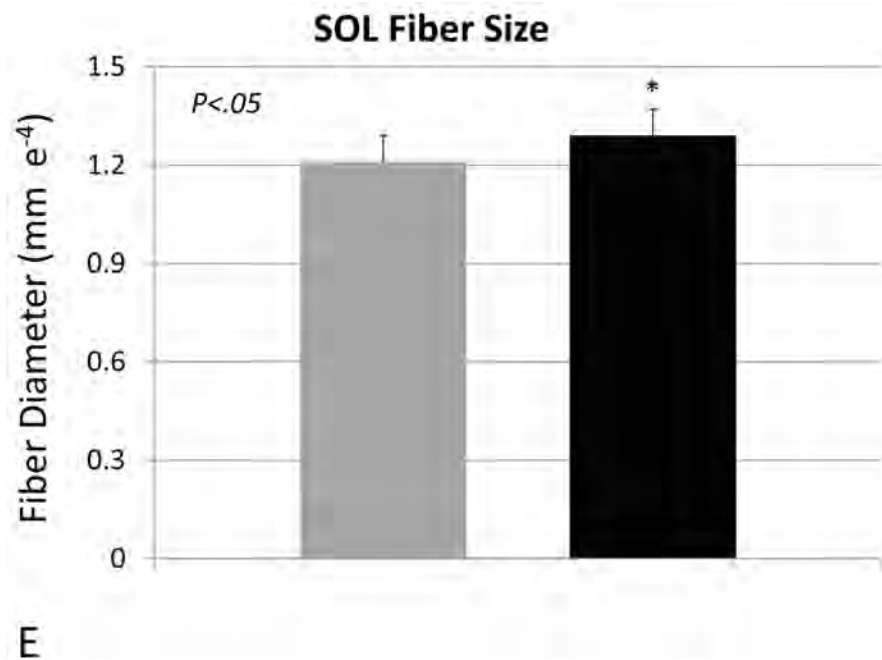
Tibias Anterior Mass

A

B

EDL Fiber Size

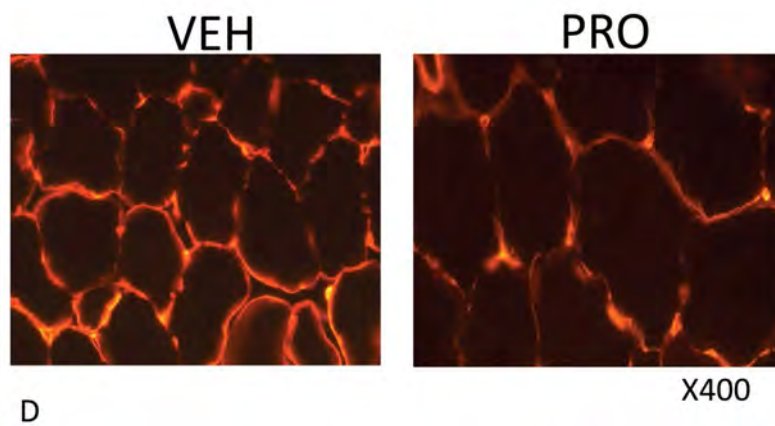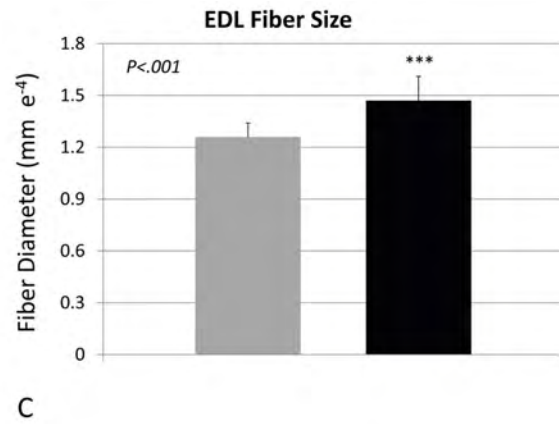
VEH   PRO
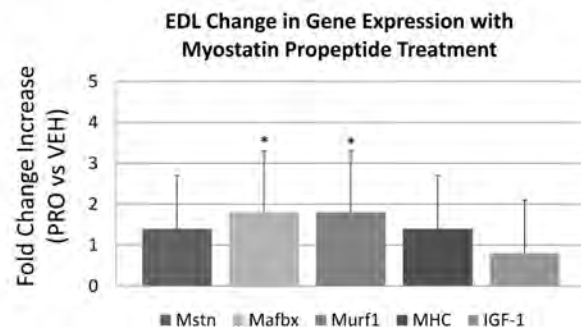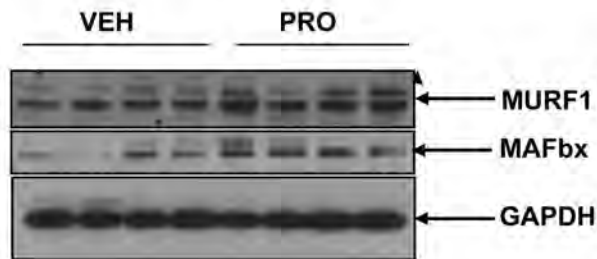
X400

D

SOL Fiber Size

E

Figure 3.
Muscle parameters for mice treated with saline (VEH) or myostatin propeptide (PRO) weekly for a period of four weeks. (A) Tibialis anterior mass, (B) tibialis anterior mass relative to body weight, (C) extensor digitorum longus fiber diameter (EDL), (D) alpha-laminin stained cryostat sections of the EDL, (E) soleus muscle fiber diameter.
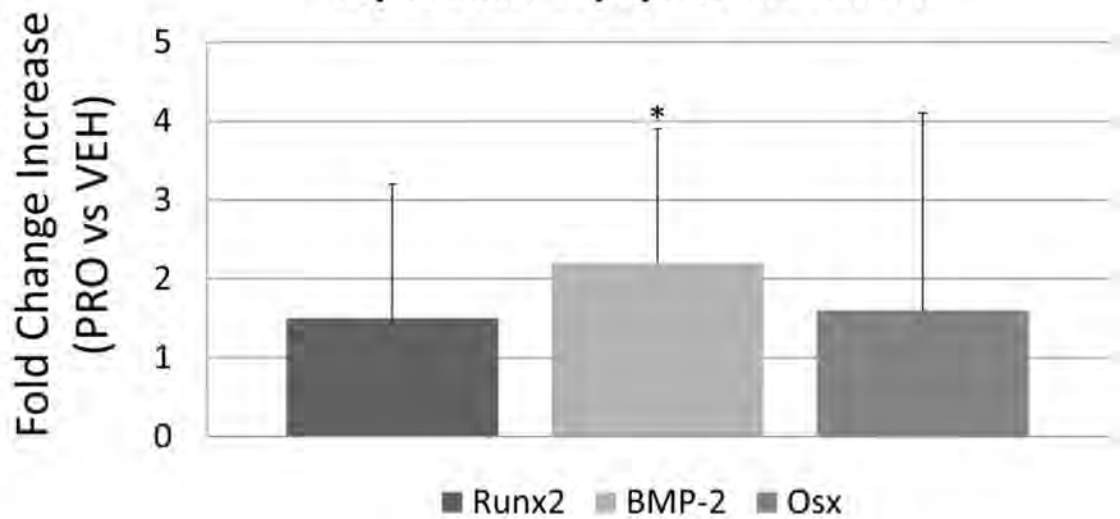
A

B

C

Figure 4.
Real-time PCR data for mice treated with saline (VEH) or myostatin propeptide (PRO) weekly for a period of four weeks showing increased expression of Murf1 and Mafbx in PRO-treated mice (A), Western blot showing similar increases in Murf1 and Mafbx with PRO-treatmend (B), and increased expression of BMP-2 in mice treated with propeptide (C). *P<.05.

Table 1

Nucleotide sequences of mouse primers used for RT-PCR

Gene	Primer	Reference/Accession Number
GAPDH	CAT GGC CTC CAA GGA GTA AGA GAG GGA GAT GCT CAG TGT TGG	M32599
18S	AGT GCG GGT CAT AAG CTT GC GGG CCT CAC TAA AC CAT CCA	V00851
β-actin	GTT TGA GAC CTT CAA CAC CCC GTG GCC ATC TCC TGC TCG AAG TC	Meredith et al 2011[*]
Mstn	ACT GGA CCT CTC GAT AGA ACA CTC ACT TAG TGC TGT GTG TGT GGA GAT	NM_010834 2
IGF-1	CAG ACA GGA GCC CAG GAA AG AAG TGC CGT ATC CCA GAG GA	NM_184052
MHC	ACA GTC AGA GGT GTG ACTC AGC CG CCG ACT TGC GGA GGA AAG GTG C	NM_001099635
Murf1	GGAGCAGCTGGAAAAGTCCACC AGCTGCTTGGCACTTGAGAGGA	NM_001039048.2
Mafbx	CAGCTTCGTGAGCGACCTC GGCAGTCGAGAAGTCCAGTC	NM_026346
BMP-2	TGT TTG GCC TGA AGC AGA GA TGA GTG CCT GCG GTA CAG AT	NM_007553 2
RUNX-2	GGA AAG GCA CTG ACT GAC CTA ACA AAT TCT AAG CTT GGG AGG A	NM_009820
Osx	ACT ACC CAC CCT TCC CTC AC ACT AGG CAG GCA GTC AGA CG	AY803733

[*] Meredith ME, Harrison FE, May JM. Differential regulation of the ascorbic acid transporter SVCT2 during development and in response to ascorbic acid depletion. Biochem Biophys Res Commun. 2011 Nov 4;414(4):737–42.

Table 2

microCT and biomechanical testing of the proximal tibia for mice treated with saline (VEH) or myostatin propeptide (PRO; 20 mg/kg).

Parameter	VEH (n=14)	PRO (n=15)	p value
BMD	1.43±0.06	1.38±0.05	.01
BV/TV	6.67±2.37	6.14±2.16	.24
Tb. Th	0.11±0.02	0.11±0.01	.47
Tb. N	0.59±0.14	0.54±0.16	.23
Fu (kg)	2.21±.40	2.18±.34	.39
U (kg/um^2)	740.6±417 5	670 3±309	.31
S (g/um)	4.6±2.0	4.7±2.0	.44

BMD=bone mineral density, BV/TV=bone volume relative to total volume, Tb.Th=trabecular thickness, Tb.N=trabecular number, Fu=ultimate force, U=energy-to-fracture, S=stiffness.

Table 3

Bone histomorphometry data for the distal femur of mice treated with saline (VEH) or myostatin propeptide (PRO; 20 mg/kg).

Parameter	VEH (n=15)	PRO (n=14)	p value
N.Ob/BS	27.26±17.49	25.09±9.31	.14
MS/BS	0.41±0.17	0.43±0.13	.34
N.Oc/BS	6.33±2.61	6.18±3.82	.38

N.Ob/BS=osteoblast number per bone surface, MS/BS=mineralizing surface (single-label) relative to bone surface, N.Oc/BS=osteoclast number per bone surface.